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Limited Chemical Structural Diversity Found to Modulate Thyroid Hormone Receptor in the Tox21 Chemical Library

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Background: Thyroid hormone receptors (TRs) are critical endocrine receptors that regulate a multitude of processes in adult and developing organisms, and thyroid hormone disruption is of high concern for neurodevelopmental and reproductive toxicities in particular. To date, only a small number of chemical classes have been identified as possible TR modulators, and the receptors appear highly selective with respect to the ligand structural diversity. Thus, the question of whether TRs are an important screening target for protection of human and wildlife health remains.

Objective: Our goal was to evaluate the hypothesis that there is limited structural diversity among environmentally relevant chemicals capable of modulating TR activity via the collaborative interagency Tox21 project.

Methods: We screened the Tox21 chemical library (8,305 unique structures) in a quantitative high-throughput, cell-based reporter gene assay for TR agonist or antagonist activity. Active compounds were further characterized using additional orthogonal assays, including mammalian one-hybrid assays, coactivator recruitment assays, and a high-throughput, fluorescent imaging, nuclear receptor translocation assay.

Results: Known agonist reference chemicals were readily identified in the TR transactivation assay, but only a single novel, direct agonist was found, the pharmaceutical betamipron. Indirect activation of TR through activation of its heterodimer partner, the retinoid-X-receptor (RXR), was also readily detected by confirmation in an RXR agonist assay. Identifying antagonists with high confidence was a challenge with the presence of significant confounding cytotoxicity and other, non-TR-specific mechanisms common to the transactivation assays. Only three pharmaceuticals—mefenamic acid, diclazuril, and risarestat—were confirmed as antagonists.

Discussion: The results support limited structural diversity for direct ligand effects on TR and imply that other potential target sites in the thyroid hormone axis should be a greater priority for bioactivity screening for thyroid axis disruptors. https://doi.org/10.1289/EHP5314

Introduction

Thyroid hormones are present in numerous tissues, including brain, pituitary, heart, fat, liver, and bone and regulate many processes, from metabolic and cardiac output rate to neurodevelopment (Cioffi et al. 2018; Duncan Bassett and Williams 2018; Gilbert et al. 2012; Oetting and Yen 2007; Williams 2008; Yen 2001; Zoeller et al. 2007). Thyroid hormones, specifically triiodothyronine (T3), predominantly exert their genomic action via interaction with thyroid hormone receptor (TRs), a family of nuclear receptor transcriptional factors including TRα1, β1, β2, and β3, expressed in a specific pattern during development and adulthood, based on their regulatory function (Cheng et al. 2010; Tancevski et al. 2011). The gene targets of the transcriptional action of TRs continue to be elucidated, including reports of genes in the brain, heart, and liver (Grijota-Martínez et al. 2011; Cheng et al. 2010; Govindan et al. 2009; Lonard et al. 2007) that demonstrate the broad physiological relevance of TR signaling at all life stages. TRβ1 is present in many tissues but is most highly expressed in liver, whereas TRβ2 is highly expressed in the anterior pituitary (Yen 2001) and is thought to be a primary determinant of hypothalamic–pituitary–thyroid axis regulation (Williams 2008). TRα1 is highly expressed in neurons (Wallis et al. 2010; Yen 2001) during fetal development, with decreased expression in the weeks following birth to coincide with dramatic increases in TRβ1, suggesting that a developmental pattern of TR isoforms expression is related to receptor-specific regulation of genes for neurodevelopment (Yen 2001). Thus, interest in screening for xenobiotics that may modulate TR is underscored by the breadth of physiology controlled by these receptors.

TRs demonstrated a stringent, selective binding profile in medicinal chemistry and X-ray crystallographic receptor studies, suggesting that only chemicals with high homology to thyroid hormones will act at this regulatory node (Ribeiro et al. 1998; Wagner et al. 1995). TRs form homodimers and heterodimers with other nuclear receptors, in particular the retinoid-X receptor (RXR), forming a regulatory complex (Araki et al. 2005; Burris et al. 2013), and recruit co-activators and co-repressors in order to interact with transcriptional response elements upstream of TR-regulated genes (Cheng et al. 2010; Govindan et al. 2009; Lee and Yen 1999). TR modulators have been developed as potential therapeutics, including the TRβ isoform-selective synthetic agonists GC-1 and KB2115 (Berkenstam et al. 2008; Chiellini et al. 1998) and NH-3 as a TRβ antagonist (Chiellini et al. 2002; Lim et al. 2002) but are limited in number and structural diversity.

In vitro assays are available to demonstrate that some non-pharmaceutical, environmental chemicals can interact with TRs and support more extensive evaluation of such compounds (DeVito et al. 1999; Murk et al. 2013; Zoeller 2005). The *in vitro* approaches used included several nuclear TR transactivation

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assays: cell lines with endogenous TRs and stable luciferase reporter genes regulated by TR-responsive promoters; stable reporter gene assays in cell lines expressing specific, recombinant TR isoforms; cell lines co-transfected with a specific GAL4-TR expression vector and a corresponding upstream activation sequence (UAS); transiently transfected versions of these assays; and stable reporter assays in yeast (Murk et al. 2013). Examples of modulators identified in receptor-reporter assays include hydroxylated polychlorinated biphenyls (OH-PCBs) and hydroxylated polybrominated diphenyl ethers (OH-BDEs) as TR agonists and amiodarone and sodium arsenite as antagonists (Freitas et al. 2011; Norman and Lavin 1989). In addition, there are several conflicting reports on the *in vitro* receptor-mediated activity of bisphenol A (BPA) and its halogenated analogs, including tetrabromobisphenol A and tetrachlorobisphenol A. These chemicals appear to be weak TR antagonists with some potential agonist-like behavior at lower concentrations similar to the effects of selective estrogen receptor modulators on cell proliferation (Freitas et al. 2011; Kitamura et al. 2002; Moriyama et al. 2002; Schriks et al. 2006). Miyazaki et al. (2008) and Ibhazehiebo et al. (2011) explained weak suppression of TR-mediated transcription by nondioxin-like PCBs and polybrominated bisphenols as caused by dissociating TR from the TR response element (TRE) although coregulator recruitment was unaffected. Kollitz et al. (2018) demonstrated T3-competitive binding of halogenated bisphenols and diphenyl ethers to human and zebrafish TRβ but did not examine functional activity. Several classes of substances were identified previously as interacting with TRs in a HepG2 cell transactivation assay for human TRα and TRβ, but with limited efficacy, including a variety of food constituents, that is, genistein, silymarin, and xanthohumol; estrogenic plasticizers including 4-nonylphenol and BPA; ultraviolet-blocking chemicals benzophenone-2 and -3; and the pesticides linuron and procymidone (Hofmann et al. 2009). Thus, it is important to screen a library that includes environmentally relevant chemicals for potential endocrine-disrupting interactions on TR functional activity and characterize the potential ligands identified to evaluate the hypothesis that the TR is likely not a primary target for xenobiotic disruption of thyroid homeostasis.

This hypothesis fits within the objectives of the U.S. Environmental Protection Agency (U.S. EPA) Endocrine Disruption Screening Program (EDSP) to screen pesticidal actives and inerts and chemicals present in the environment for their potential to disrupt endocrine function in both humans and wildlife. We utilized the Tox21 consortium (Kavlock et al. 2009), which employs a large set of high-throughput screening (HTS) assays with the goal of profiling thousands of environmentally relevant chemicals for their potential to perturb biological pathways, including endocrine function. The rat pituitary GH3 TR reporter gene cell line, expressing endogenous TRα and TRβ, was used to screen the Tox21 8,305-chemical library, followed by orthogonal assays that were then used to characterize the potential agonists and antagonists identified. These data provide evidence to evaluate the relative contribution of TR disruption to xenobiotic-induced adverse outcomes for thyroid axis function and to further inform development of predictive tools for thyroid signaling disruption.

Methods

Chemical Library Screened

The Tox21 chemical library contained 10,496 samples and 8,305 unique structures at the time of this screen. The compound classes include industrial chemicals, sunscreen additives, flame retardants, pesticides and selected metabolites, plasticizers, solvents, food additives, natural product components, drinking-water disinfection by-products, preservatives, therapeutic agents, and chemical synthesis by-products. Identity and purity quality control analysis of the entire library was conducted using a tiered approach beginning with a high-throughput high-performance liquid chromatography system. Results for the library are available at https://tripod.nih.gov/tox21/samples with results for specific compounds also shown in this manuscript. Note that these techniques are high-throughput analytical methods and should be considered supportive, but not necessarily definitive, data for chemical identification and purity for the tested sample solutions in dimethyl sulfoxide (DMSO).

TR Screening Assays

The assays used in this study, including cell line, primary function of the assay in the current work, and its unique assay end point identifier within the U.S. EPA's invitrodb_v2 database (https://doi.org/10.23645/epacomptox.6062623.v1) are listed in Table 1 and the project workflow in Figure S1.

GH3-TRE screening assays. **Cell line and culture.** The development of the GH3-TRE-Luc cell line for assays used in the primary screening was previously described (Freitas et al. 2011, 2014). Briefly, a thyroid hormone receptor-regulated luciferase reporter containing two thyroid hormone DR4 response elements upstream of an SV40 minimal promoter driving expression of a modified firefly luciferase reporter was stably cloned into the rat pituitary tumor cell line, GH3. The GH3-TRE-Luc cell line was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

Table 1. Assay names (aenm) and assay end point identification (aeid) values used in the text and invitrodb database together with mode and purpose of assay.

Assay short name	invitrodb: aenm	invitrodb: aeid	Cell line	Assay mode	Function
GH3-TRE-Ag	TOX21_TR_LUC_GH3_Agonist	803	GH3-TRE-Luc	Agonist	Primary qHTS
GH3-TRE-Antag	TOX21_TR_LUC_GH3_Antagonist	804	GH3-TRE-Luc	Antagonist	Primary qHTS
GH3-TRE-Via	TOX21_TR_LUC_GH3_Antagonist_viability	805	GH3-TRE-Luc	Viability	Cytotoxicity
GH3-TRE-Ag-Followup	TOX21_TR_LUC_GH3_Agonist_Followup	2226	GH3-TRE-Luc	Agonist	Confirmation
GH3-TRE-Antag-Followup	TOX21_TR_LUC_GH3_Antagonist_Followup	2227	GH3-TRE-Luc	Antagonist	Confirmation
TRb-bla	TOX21_TRB_BLA_Antagonist_Followup_ratio	2240	TRβ-UAS-bla HEK 293T	Antagonist	Specificity
RXRa-bla-Ag	TOX21_TR_RXR_BLA_Agonist_Followup_ratio	2253	RXRα-UAS-bla HEK 293T	Agonist	Specificity
RXRa-bla-Antag	TOX21_TR_RXR_BLA_Antagonist_Followup_ratio	2257	RXRα-UAS-bla HEK 293T	Antagonist	Specificity
RXRa-Via	TOX21_TR_RXR_BLA_Antagonist_Followup_viability	2258	RXRα-UAS-bla HEK 293T	Viability	Cytotoxicity
TRa-coa	TOX21_TRA_COA_Agonist_Followup_ratio	2230	NA	Agonist	Orthogonal
TRb-coa	TOX21_TRB_BLA_Agonist_Followup_ratio	2236	NA	Agonist	Orthogonal
GFP-GR-TRb	NA	NA	GFP-GR-TRβ MCF7	Agonist and antagonist	Orthogonal

Note: Ag, agonist; Antag, antagonist; bla, beta-lactamase; coa, coactivator; GFP, green fluorescent protein;GH3, rat pituitary cell line; GR, glucocorticoid receptor; HEK 293T, human embryonic kidney cell line; LUC, luciferase; MCF7, human breast cancer cell line; NA, not applicable; qHTS, quantitative high-throughput screen; RXRa, retinoid X receptor alpha; TRa, thyroid hormone receptor alpha; TRb, thyroid hormone receptor beta; TRE, thyroid hormone receptor response element; UAS, upstream activating sequence; Via, viability.

(DMEM/F-12) supplemented with 10% fetal bovine serum (FBS; Hyclone, SH30071.03) and 100 U/mL penicillin-100 μg/mL streptomycin (Life Technologies).

Primary screen. For the primary quantitative high-throughput screen (qHTS) assays, the growth medium from the GH3-TRE-Luc cell culturing flask was replaced with assay medium [DMEM/F-12 medium supplemented with 10 ng/mL sodium selenite, 10 μg/mL human apo-transferrin, 10 μM ethanolamine, 10 μg/mL insulin, and 500 μg/mL bovine serum albumin (BSA)] overnight prior to the assay. The next day, cells were seeded at 1,500/well in 5 μL (agonist mode) and 4 μL (antagonist mode) of the assay medium in 1,536-well white/solid plates (Greiner Bio-One North America) using a Multidrop Combi (Thermo Fisher Scientific) dispenser. Chemical samples were serially diluted in DMSO, yielding 15 concentrations for testing. After 5-h incubation at 37°C/5% carbon dioxide (CO_2) for cell attachment, 23 nL of serially diluted compounds (yielding 92 μM to 5 nM final assay concentration) and positive control (T3; agonist format), DMSO only (antagonist format) were transferred to the assay plates using a Pintool station (Wako). To screen compounds that antagonize T3-induced transactivation of TR, an extra 1 μL of T3 was added using a BioRAPTER Flying Reagent Dispenser™ (FRD™; Aurora Discovery) on the top of the cell/compound mixtures to achieve a final agonist concentration of 1.0 nM. The assay plates were incubated at 37°C/5% CO_2 for 24 h. For the antagonist mode, a cell viability assay was run in parallel by adding 1 μL/well CellTiter-Fluor reagent (Promega Corporation) using an FRD™, and after 30-min incubation at 37°C/5% CO_2, the fluorescence intensity was measured using a ViewLux plate reader (PerkinElmer). For the luciferase reporter gene assay, 5 μL/well (agonist format) and 4 μL/well (antagonist format) ONE-Glo reagent (Promega Corporation) was added using an FRD™, and after 30-min incubation at room temperature, the luminescence intensity was measured using a ViewLux plate reader. Data were expressed as relative fluorescence units (cell viability assay) and relative luminescence units (luciferase reporter assay).

Confirmatory screen. Samples were selected based on an active hit call in the primary qHTS assay, agonist or antagonist format, together with the availability of sample from reserved chemical stocks for the Tox21 library for retesting in the GH3-TRE-Luc assay using the same format at the primary screen. Samples were retested in parallel with TRα and TRβ assays in the mammalian one-hybrid assays to assess activity against human TR ligand-binding domains.

Mammalian one-hybrid beta-lactamase screening assays. **Specificity screening for TRβ**. The GeneBLAzer™ TRβ-UAS-bla HEK 293T assay kit for human TRβ (Thermo Fisher Scientific; catalog no. K1684) was used as a secondary assay for specificity (TRβ-bla). This assay contained the ligand-binding domain (LBD) of the human TRβ fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer™ UAS-bla HEK 293T cell line. The reporter gene, β-lactamase, is under the transcriptional control of a UAS that binds the GAL4 DNA-binding domain. A cell viability assay was run in parallel for these cells as well using the CellTiter-Glo viability assay kit.

TRβ-bla cells were dispensed at 3,000 cells/well in 5 μL of the assay medium containing phenol red–free DMEM with 2% charcoal-stripped FBS in 1,536-well black-wall/clear-bottom plates using an FRD™. After 5-h incubation at 37°C/5% CO_2 for cell attachment to the well bottom, 23 nL of follow-up compounds dissolved in DMSO, 11 concentrations in triplicate, and the positive control (T3; agonist format) were transferred to the assay plates using a Pintool station. The assay plates were incubated at 37°C/5% CO_2 for 18 h. One microliter LiveBLAzer™ FRET-B/G CCF4-AM substrate was added to each well using an FRD™. After 2-h incubation at room temperature, the fluorescence intensity was measured using an Envision plate reader. Data were expressed as the ratio of 460 : 530 nm. The cytotoxicity of the compounds was measured in the same plates by adding 4 μL of CellTiter-Glo reagent to each well and incubating the plates at room temperature for 30 min. The luminescence intensity was measured using a ViewLux plate reader.

Specificity screening for RXR. To assess whether activity at the RXR portion of the TR:RXR heterodimer may have contributed to the GH3-TRE-Luc signal, a GeneBLAzer™ RXRα-UAS-bla HEK 293T assay (RXRα-bla) was also run (Thermo Fisher Scientific; catalog no. K1697). RXRα-bla cells were seeded at 2,500/well in 6 μL of the assay medium containing phenol red–free DMEM with 2% charcoal-stripped FBS in 1,536-well black-wall/clear-bottom plates (Greiner Bio-One North America) using a Multidrop Combi dispenser. After 5-h incubation at 37°C/5% CO_2 for cell attachment, 23 nL of follow-up compounds dissolved in DMSO, 11 concentrations in triplicate, and the positive control (9-*cis* retinoic acid) were transferred to the assay plates using a Pintool station. The assay plates were incubated at 37°C/5% CO_2 for 16 h. One microliter of LiveBLAzer™ FRET-B/G CCF4-AM substrate (Life Technologies) was added to each well using an FRD™. After 2-h incubation at room temperature, the fluorescence intensity was measured using an Envision plate reader (PerkinElmer). Data were expressed as the ratio of 460 : 530 nm.

Orthogonal assays. **Coactivator recruitment assays (TRa-coa and TRb-coa).** The LanthaScreen™ TR-FRET TRβ and TRα coactivator assay kits were used to determine TR activity in a cell-free functional assay in a 1,536-well qHTS format. Briefly, 4 μL mixture of the receptor LBDs expressed as glutathione *S*-transferase (GST) fusion proteins (2.5 nM for TRα and 5.0 nM for TRβ) and BSA (0.10%; Sigma-Aldrich) were incubated with 23 nL of control or test compound. A time-resolved fluorescence resonance energy transfer (TR-FRET) signal was indicative of coactivator recruitment using a 2 μL mixture of fluorescein-labeled SRC-2 peptide (200 nM) and terbium-labeled anti-GST antibody (2 nM), which transferred resonance energy to the fluorescein tag on the coactivator peptide following recruitment to the receptor. Fluorescence emission ratios (520 : 495 nm) were collected by an Envision plate reader (excitation at 340 nm and emissions at 495 and 520 nm), and the measured values were normalized to 0% activity using DMSO-only wells and 100% activity using T3 control wells.

Nuclear translocation assay (GFP-GR-TRβ). Ligand-induced cytoplasm-to-nuclear translocation/stabilization of the TR was determined by using a variation of a glucocorticoid receptor (GR) nuclear translocation assay (Htun et al. 1996; Stavreva et al. 2016). Briefly, a GFP-GR-TRβ chimeric receptor was constructed by fusing the human GR N-terminus, DNA-binding domain and the hinge region to human TRβ ligand-binding domain. The chimeric GFP-tagged receptor was stably expressed in MCF7 Tet-off Advanced cell line (Clontech). Cells plated in duplicates on a 384-well plate were treated with compounds or a diluent as control for 3 h, fixed with paraformaldehyde, the nuclei stained with DRAQ5™ (abcam) or 4′,6-diamindino-2-phenylindole (DAPI) and cells imaged on the PerkinElmer Opera quadruple-enhanced high-sensitivity high-content screening platform with fully automated confocal image collection. An image analysis pipeline was customized using the Columbus software (PerkinElmer) to automatically segment the nucleus using the DAPI channel and then construct a ring region (cytoplasm) around the nucleus mask for each cell in the digital micrographs. Translocation was calculated as a ratio of the mean GFP-GR-TRβ intensity in nucleus and cytoplasm, and each value was further normalized to the value for the control (i.e., DMSO) sample on the same plate.

Data Analysis for Tox21 Assay Data

Each chemical in the qHTS assays from Tox21 was tested independently three times, with internal replication of chemicals with multiple samples in the library using 15-concentrations (for GH3-TRE-Luc assays) or 11-concentrations (for the mammalian one-hybrid BLA assays and coactivator recruitment assays). The data set was then corrected using an in-house pattern correction algorithm and normalized to controls (Wang and Huang 2016). Curve-fitting and hit-calling on the corrected data used the U.S. EPA's ToxCast data pipeline (tcpl) (tcpl R package, version 1.2.2) (Filer et al. 2017). Curve-fitting based on the winning model (gain-loss, Hill, or constant) is summarized in level 5 of the ToxCast database, invitrodb_v2, including values such as the hit call (hitc), 50% activity concentration (AC_{50}) and maximum efficacy expressed as a percent of the positive control response (EMax). A positive hitc (hitc = 1) indicates that the top of the winning model for curve surpassed the user-defined cutoff for minimum efficacy to be called a positive. The cutoff for these assays was defined statistically as the greater of six times baseline median absolute deviation (bmad) or a 20% change from control. The bmad, cutoff, number of tested chemicals as defined by a chemical identification (chid), and the resultant active number and active percent in the assay are summarized in Excel Table S1. Results were reviewed for potentially bad curve fits, primarily keyed by the data quality flags accompanying the tcpl analysis, and hitc and AC_{50} adjusted manually, if required. Inactive samples were arbitrarily set to AC_{50} = 1,000 μM, and the resulting data set was used for analysis. For the TR assays in agonist mode, 1 nM T3 was the positive control and DMSO the neutral control. For the TR assays in antagonist mode, 1 nM T3 was the neutral control and DMSO the positive control, that is, DMSO vehicle defined the minimum response in the assay. The complete tcpl curve-fitting results as well as manual review for the qHTS agonist, antagonist, and corresponding cell viability assays are presented in Excel Table S2 and follow-up transactivation assays in Excel Table S3. For the RXR agonist assays, 5 μM 9-*cis* retinoic acid was used as the positive control and DMSO as the neutral control. For the RXR antagonist assays, 100 nM 9-*cis* retinoic acid was used as the neutral control and DMSO was used to define the minimum response in the assay. Data output from tcpl was than combined and summarized at the chemical level where multiple samples of the same chemical structure were present. All source data are available from the iCSS ToxCast dashboard version 2 (https://comptox.epa.gov/dashboard) and the invitrodb_v2 database (https://doi.org/10.23645/epacomptox.6062623.v1).

Additional Context for Curating TR Agonists and Antagonists

Cell viability analysis. Eighteen cell viability assays using different technologies and cell types were performed on the Tox21 library compounds as part of the Tox21 project (Judson et al. 2016) (see Excel Table S4). The percentage resulting in hitc = 1 and the median and lowest AC_{50} were determined. These results were used with the other assay results described here in a weight-of-evidence approach to help with selecting candidate TR antagonists for follow-up testing.

Tanimoto similarity score on indigo fingerprints. All compounds identified as agonists were searched using batch mode against the 761,000 structures in the U.S. EPA CompTox Chemicals Dashboard to identify structurally similar compounds (https://comptox.epa.gov/dashboard/dsstoxdb/batch_search). Similarity is based on a Tanimoto score derived from Indigo Toolkit structural fingerprints using the 1.7.10-dev6.3 linux64 version of the Bingo PostgreSQL Cartridge with the default Indigo fingerprint (http://lifescience.opensource.epam.com/indigo/). The similarity search tool returned 130 compounds with a Tanimoto score of >0.8. The CAS registration number (CASRN) for these compounds was used to query the Tox21 chemical library to identify potential false negatives from the qHTS assay. The three compounds with the highest confidence results for antagonists were also searched in DSSTox for related substances. There were 204 compounds found with Tanimoto scores >0.8; however, none were included in the Tox21 screening library.

Active rate comparison between receptors. To compare active calls between receptors, we compared a subset of the Tox21 library chemicals screened in multiple estrogen receptor and androgen receptor assays that were used to build predictive systems biology models for agonist and antagonist modes, known as the ToxCast pathway models (Judson et al. 2015; Kleinstreuer et al. 2017). We first removed chemicals classified as pharmaceuticals because these sets of chemicals are biased toward drugs targeting estrogen receptors and androgen receptors. We then used the recommended model values of ≥0.1 to define actives for both receptors and both modes. For TR, we cross-referenced these same chemicals for testing against TR and used candidate chemicals in Tables 2 and 3 to define active rates.

Results

Agonists

Complete results from both GH3-TRE qHTS agonist, antagonist, and viability mode for the 8,305 compounds are provided in Excel Table S2. For the agonist mode, 35 active compounds were considered active (hitc = 1) following the automated tcpl curve-fitting algorithm. We reviewed all 35 active fits manually to identify possible false positives resulting from obvious outlier data as well as potential false negatives among any other curves that had an inactive hitc (hitc = 0) but demonstrated an EMax of 50% or greater. This resulted in 28 confirmed active compounds ranging in potency from <1 nM (lowest concentration tested) to 667 μM (see Excel Table S5). Together with some potential false negatives, these chemicals, totaling 84 in all, were screened for activity in a series of assays for confirmation in the GH3-TRE-Ag assay; specificity screening in the TRβ-UAS-bla HEK 293T and RXRα-UAS-bla HEK 293T mammalian one-hybrid assays; viability assays for the HEK293T parental cell line; and orthogonal screening in two TR:coactivator recruitment assays, TRa-coa and TRb-coa. Results, summarized in Table 2, with more details in Excel Table S6, showed 16 of the original active compounds repeated as actives in the GH3 assay, along with 5 additional compounds that originally had hitc = 0 but significant efficacy. Behavior across the characterization assays showed that the compounds could be clustered into several mechanistic categories—full agonists, indirect TR agonists/RXR agonists, and weak/ambiguous actives—as described below.

Full Agonists. Eight compounds were active in all three of the TR transactivation assays, active in both coactivator recruitment assays, inactive in the RXR assay (or only showing weak activity at greater than a 5-fold higher concentration compared with TR), and no cytotoxicity was detected in the range of the reporter activity. The average EMax in the TR transactivation assays in the primary and follow-up GH3 assay for these full agonists (excluding tromethamine hydrochloride) was 102 ± 19% of the response of the positive control, T3. This supports full agonist pharmacological behavior at the receptor for these ligands. An example characteristic of results for a full agonist, betamipron, is shown in Figure 1A. It showed concentration-dependent activity in the GH3-TRE-Luc assay as an agonist in the rat GH3 cell for TR, in the TRβ-bla as an agonist for the human TRβ ligand-

Table 2. Activities associated with agonist active chemicals.

Chemical name	GH3-TRE-Ag AC50 (μM)	GH3-TRE-Ag-Followup AC50 (μM)	TRa-coa-Ag AC50 (μM)	TRb-coa-Ag AC50 (μM)	TRb-bla-Ag AC50 (μM)	RXRa-bla-Ag AC50 (μM)	GH3-TRE-Via AC50 (μM)	Global cytotoxicity AC50 median (μM)	Classification
CP-634384	0.0015	0.0180	0.0029	0.0020	0.0865	44.299	>92	>92	TR-Ag
3,5,3′-Triiodothyronine	0.0011	0.0007	0.0034	0.0034	0.0009	>92	41.687	>92	TR-Ag
Levothyroxine	0.0061	0.0075	0.0340	0.1390	0.0361	1.3624	>92	>92	TR-Ag
Tetrac	0.0009	0.0060	0.2484	0.2266	0.0209	23.551	51.286	35.7	TR-Ag
3,3′,5′-Triiodo-L-thyronine	0.2183	0.5373	0.7065	1.0002	0.6415	1,000	>92	>92	TR-Ag
Tiratricol	0.000010	0.0002	0.0057	0.0047	0.0005	7.872	>92	27.0	TR-Ag
3,3′,5-Triiodo-L-thyronine sodium salt	0.0002	0.0021	0.0133	0.0126	0.0036	>92	>92	>92	TR-Ag
Betamipron	11.8412	1.6786	10.403	11.765	14.030	>92	>92	>92	TR-Ag
all *trans*-Retinoic acid	0.2336	1.1589	>92	>92	1.480	0.7878	66.737	>92	RXR-Ag
(Acryloyloxy)(tributyl)stannane	0.07351	>92	>92	>92	>92	0.1410	0.508	1.0	RXR-Ag
Acitretin	4.3272	0.9399	>92	9.701	>92	0.5639	>92	>92	RXR-Ag
13-*cis* Retinoic acid	0.1328	0.0348	>92	>92	>92	0.0236	81.283	46.3	RXR-Ag
9-*cis* Retinoic acid	4.1796	2.0677	>92	>92	>92	1.848	>92	>92	RXR-Ag
Sincalide	12.376	8.588	>92	>92	>92	10.496	>92	>92	RXR-Ag
Clofoctol	1.374	3.004	8.920	8.928	>92	6.796	8.035	16.7	RXR-Ag
Bexarotene	0.0077	0.0170	>92	>92	>92	0.0053	10.351	23.9	RXR-Ag
Equilin	3.4083	>92	>92	>92	>92	0.9860	85.114	>92	RXR-Ag

Note: AC50, concentration producing 50% activation of the receptor; Ag, agonist; bla, beta-lactamase; coa, coactivator; GH3, rat pituitary cell line; RXRa, retinoid X receptor alpha; TR, thyroid hormone receptor; TRa, thyroid hormone receptor alpha; TRb, thyroid hormone receptor beta; TRE, thyroid hormone receptor response element; Via, viability.

binding domain, in the TRa-coa and TRb-coa biochemical assays showing activation of receptor recruitment of coactivator peptide, and lack of activity against the human RXRα receptor assay. The structures of this group of full TR agonists are shown in Figure S2A and consist of known TR ligands T3 (3,5,3′-triiodothyronine; two salt forms active); 3,3′,5′-triiodo-L-thyronine and the prohormone T4 (3,3′,5,5′-tetraiodothyroacetic acid); synthetic analogs levothyroxine and tiratricol; and an oxamic acid–derivative pharmaceutical compound, CP-634384, developed as a TR ligand (Stanton et al. 2000). The other compounds in this category are betamipron, a drug used to reduce nephrotoxicity by blocking kidney uptake of an antibiotic and not previously described as a TR agonist (Hirouchi et al. 1994) and tromethamine hydrochloride (structure not shown), an unexpected finding for a low-molecular-weight compound commonly employed as a biochemical buffer. The analytical chemistry quality control for the tromethamine hydrochloride sample was unavailable, but three samples of the free acid version of the compound, tromethamine (CASRN 77-86-1), were completely inactive in the original TR GH3 qHTS assay and each had confirmed structures with purity >90%. Thus, the activity seen for tromethamine hydrochloride may be due to a contaminant of the sample with a potent TR agonist such as T3.

Indirect TR Agonists/RXR Agonists. Seven compounds were active in the GH3 assay but not in the TR assays in mammalian one-hybrid format nor in the TR coactivator recruitment assays. Results for the characterization of 13-*cis* retinoic acid are shown as a representative response in Figure 1B. This chemical was a partial agonist in the GH3-TRE-Ag assay (EMax of 24% of control), not active in the TRβ-bla as an agonist for the human TRβ ligand-binding domain, not active in the TRb-coa biochemical assay, but active against the human RXRα receptor in the RXRα-bla-Ag assay. All of the active structures for this class are shown in Figure S2B. They had an average EMax value for the GH3 assay of $45 \pm 26\%$. They were, however, active in the RXR transactivation assay and had average EMax values for RXR of $75 \pm 28\%$. Several of these are synthetic rexinoids (bexarotene/Targretin®, 13-*cis* retinoic acid/Accutane®) or retinoids with known RXR activity (acitretin/Soriatane®, 9-*cis* retinoic acid/Panretin®, *trans*-retinoic acid/tretinoin). Sincalide, an 8-amino acid carboxy-terminal fragment of cholecystokinin also known as CCK-8, is not known as an RXR ligand and its structure is very distinct from known agonists. Analysis of other results from Tox21 screening assays showed it inactive against four other nuclear receptors but active for two estrogen receptor transactivation assays (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID7048617#). All in this group were inactive in the mammalian one-hybrid TR assays in which heterodimerization with RXR is not necessary for transcriptional activity. Thus, these appear to be RXR agonists acting at the TR response element in the GH3 assay through a permissive heterodimerization with TR, consistent with known pharmacology (Castillo et al. 2004).

Weak/Ambiguous Actives. There were 10 inactive compounds in most or all other characterization assays beyond TR-GH3 (see Figure S2C). These mostly had low EMax values with an average of $32 \pm 11\%$. The data are inconclusive as to whether these represent very weak TR or RXR agonists, directly or indirectly, or an assay interference of unknown mechanism. One of these compounds, *N,N*-dimethyl-4-nitrosoaniline, was found to be a luciferase inhibitor, a mechanism that can paradoxically increase signal in luciferase reporter gene assays due to stabilization of the luciferase enzyme, although the lack of additional luciferase inhibitors behaving as agonists suggests this assay was not sensitive to such a mechanism (Thorne et al. 2010). Another, clofoctol, had activity in the coactivator recruitment assays and

Table 3. Activities associated with antagonist active chemicals.

Chemical name	GH3-TRE-Ag AC50 (μM)	GH3-TRE-Antag AC50 (μM)	GH3-TRE-Via AC50 (μM)	Viability/antagonist ratio	Global cytotoxicity AC50 median (μM)	Active in global cytotoxicity assays (%)	Average cell number/field in nuclear translocation assay	Nuclear/cytoplasm ratio (EMax)
Mefenamic acid	1,000	19.50	1,000	51.3	50.80	4.5	2,472	3.38
Risarestat	1,000	5.63	1,000	177.6	16.36	11.9	2,349	3.31
Carfilzomib	1,000	0.025	0.037	1.5	0.46	64.4	1,642	3.28
Diclazuril	1,000	4.70	1,000	212.8	32.98	33.3	2,608	2.69
Omacetaxine mepesuccinate	1,000	0.029	0.057	2.0	0.06	60.0	1,601	1.74
Daunorubicin	1,000	0.700	1.01	1.4	10.46	66.1	224	1.85
1-Dodecyl-3-methylimidazolium iodide	1,000	1.33	5.10	3.8	41.10	74.2	282	1.71
Manidipine dihydrochloride	1,000	7.40	9.06	1.2	18.62	28.8	2,325	1.69
4,4′-Methylenebis(*N*,*N*-diglycidylaniline)	1,000	8.03	11.10	1.4	45.26	59.1	2,095	1.69
Amlodipine besylate	1,000	4.56	2.15	0.5	NT	NT	912	1.68
Methyl red	1,000	8.79	14.59	1.7	48.54	48.5	2,101	1.68
C.I. Basic Red 9 monohydrochloride	1,000	5.15	8.80	1.7	24.17	66.7	2,197	1.67
Fludarabine	1,000	16.27	22.31	1.4	1,000	1.7	1,546	1.64
Andrographolide	1,000	24.61	19.35	0.8	NT	NT	2,333	1.64
Tomelukast	1,000	25.92	1,000	38.6	14.44	9.1	2,178	1.64
(Z,E)-Diethylstilbestrol	1,000	17.06	12.20	0.7	NT	NT	2,398	1.63
Duloxetine hydrochloride	1,000	6.22	5.69	0.9	NT	NT	1,184	1.53
5,7-Dichloro-8-hydroxy-2-methylquinoline	1,000	1.50	1.56	1.0	13.62	13.6	1,071	1.61
Rosaramicin	1,000	22.58	24.43	1.1	26.84	46.7	1,824	1.52
AMI-193	1,000	15.84	14.08	0.9	NT	NT	2,291	1.50

Note: AC50, concentration producing 50% activation of the receptor; Ag, agonist; Antag, antagonist; EMax, maximal response value; GH3, rat pituitary cell line; NT, not tested; TRE, thyroid hormone receptor response element; Via, viability.

the RXR assay (although the EMax was only 36%) but not in the one-hybrid HEK293 assay for TR. It was also a promiscuous active in other Tox21 reporter gene assays. It is an older antibiotic but more recently found to inhibit prostate cancer cell growth, possibly through activation of the unfolded protein response pathway (Wang et al. 2014). Finally, glycocholic acid is a bile acid that was active in the GH3-TRE primary screen and follow-up and inactive in all the other assays. Interestingly, it was tested in over 200 other assays in the ToxCast and Tox21 programs and found active only in the GH3-TRE assay, a TRα agonist assay run as part of a multiplexed transcription factor activation assay (ATG_THRa1_TRANS_up) and two estrogen receptor assays (ATG_ERE_CIS_up; TOX21_ERa_LUC_BG1_Agonist) (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID7048617#). The lack of activity in both the mammalian one-hybrid and the coactivator recruitment assays support this being a false positive of some type, but further investigation may be warranted.

As a means of evaluating the potential of false-negative screening results for the agonists, we used structures of the confirmed agonists to query the 761,000 structures in the DSSTox database to identify similar structures by Tanimoto score (>0.8) based on Indigo Toolkit structure fingerprints. There were 130 new compounds identified (see Excel Table S7), but only one was included in the Tox21 library, 3,5-diiodo-L-tyrosine (CASRN 300-39-0), a precursor to thyroid hormone. It was completely inactive in the GH3-TRE assay. It had a high Tanimoto score (0.89) compared against 3-5-3′-triidothyronine but lacks an entire phenol ring, which could readily explain lack of efficacy and/or affinity for TR. The DSSTox database consists of a compilation of existing lists of chemicals not only of environmental concern but also lists of compounds of interest for their bioactivity such as drugs and pharmacological probes from PubMed. Of the 130 similar compounds, only 16 were part of any of the lists specifically available on the CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard/chemical_lists) that focuses on environmental chemical inventories.

Antagonists

The antagonist mode of the GH3-TRE assay was conducted under conditions where the reporter gene was activated with an approximate EC_{85} (85% of maximal activity; 1 nM) of T3. There were 2,394 chemicals with active hit calls and calculated AC_{50} values, which were reduced to 2,375 by manual curve review (see Excel Table S8). A cell viability assay was run in parallel because of the known confounding problem of cytotoxicity resulting in false positives due to the loss-of-signal assay format (Crisman et al. 2007). Of the potential androgen receptor antagonists, 1,578 were also scored as cytotoxic in the concurrently run viability assay, 1,416 of these with less than 3-fold difference in AC_{50} in the two assays. Even with stringent rules such as requiring at least a 10-fold difference in the AC_{50} for antagonism versus cell viability, there were 812 potential TR antagonists, including compounds with well-known cytotoxic, cytostatic, or protein translation inhibition mechanisms of action. Topotecan hydrochloride, for example, a topoisomerase inhibitor and chemotherapeutic drug, showed complete inhibition of luciferase activity with an AC_{50} of 2.4 μM. However, it was not considered active in the cell viability assay, similar to other Tox21 cell viability assays (see, for example, AID 743033 in PubChem, https://pubchem.ncbi.nlm.nih.gov/bioassay/743033#section=Top).

To identify true antagonists of TR, we evaluated the ability of the candidate compounds to induce nuclear translocation of a green fluorescent protein (GFP)-tagged human GR-TRβ hybrid receptor in MCF7 cells using the known TR antagonist, 1-850, as

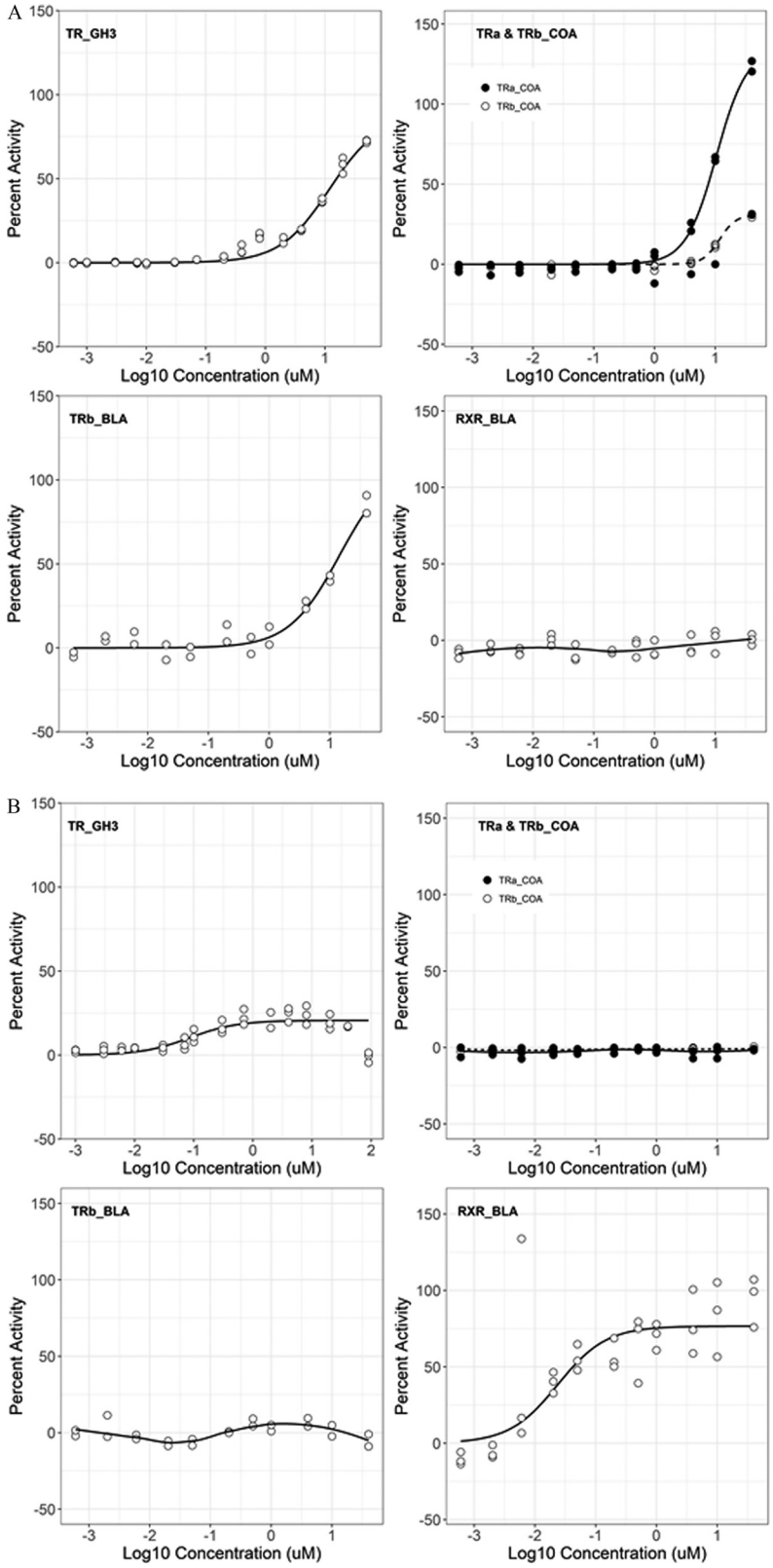

Figure 1. Example characterization of thyroid hormone receptor (TR) agonists. Candidate TR agonists were tested in concentration–response format in three reporter gene assays: 1) TR_GH3, the rat pituitary cell line used in the qHTS; 2) TRβ_HEK293, a mammalian one-hybrid format assay using the human TRβ ligand-binding domain and a β-lactamase reporter gene in HEK293 cells; and 3) RXR_HEK293, a mammalian one-hybrid format assay using the human RXRα ligand-binding domain and a β-lactamase reporter gene in HEK293 cells. The fourth assay, TRβ_SRC2, is a functional biochemical assay that measures the ligand-stimulated recruitment of a SRC2 peptide to human TRβ ligand-binding domain. Examples are provided for (A) a direct-acting agonist, betamipron, and (B) an indirect activator, 13-*cis* retinoic acid, working through RXR.

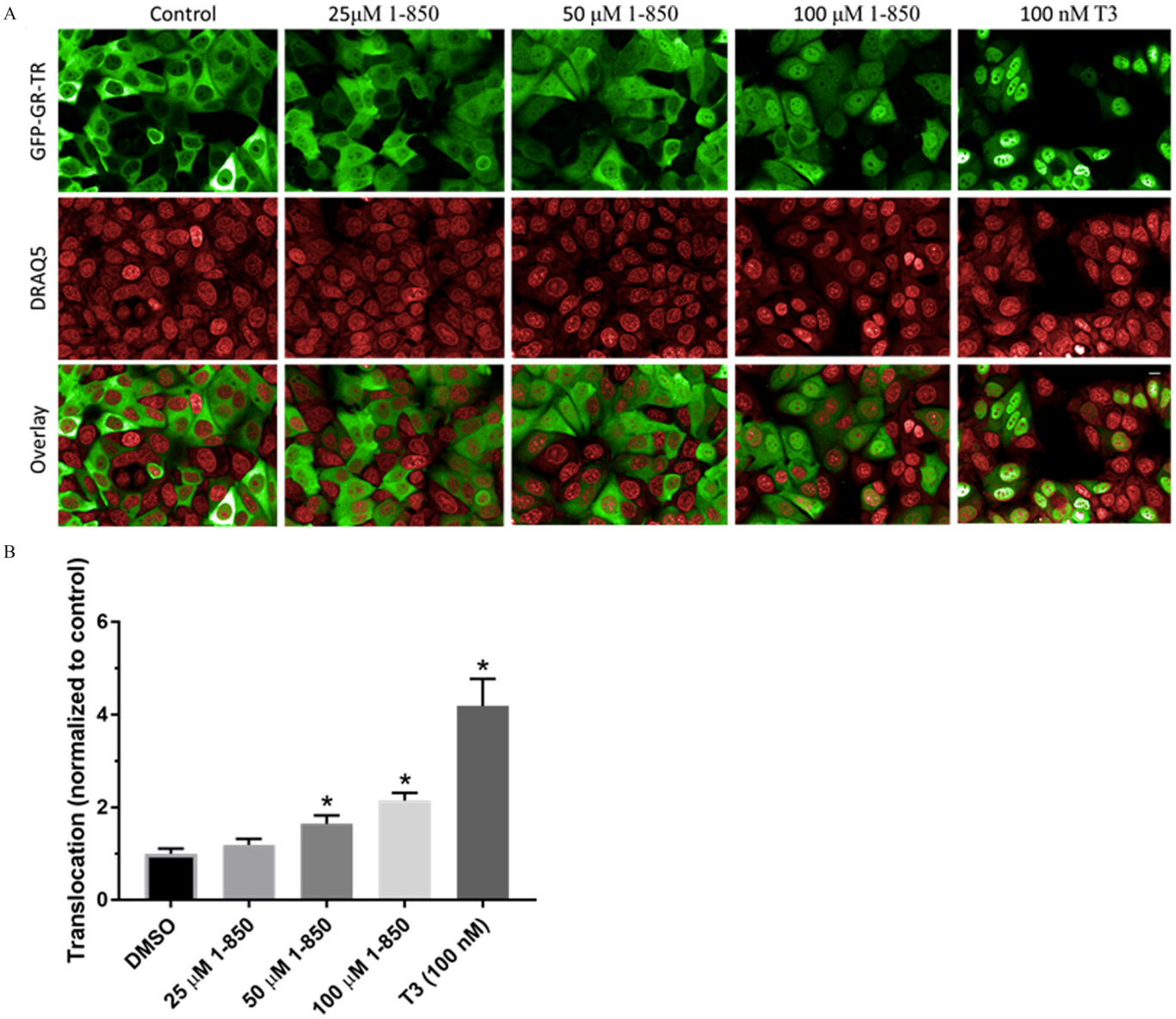

Figure 2. Stimulation of nuclear translocation of a GFP-GR-TRβ fusion protein by thyroid hormone receptor (TR) agonists and antagonists. A human MCF7 breast cancer cell line mammary adenocarcinoma cell line that expresses GFP-GR-TRβ was treated with the vehicle control [dimethyl sulfoxide (DMSO)], the positive agonist control triiodothyronine (T3) at 100 nM, or the antagonist control (1-850) at 25, 50, and 100 μM for 3 h followed by fixation, nuclear staining with DRAQ5™ (abcam) and imaging. An automated image analysis of localization was performed using the Opera (PerkinElmer) automated imaging system. (A) Representative images from the green fluorescent protein (GFP) channel, the DRAQ5™ channel, and an overlay for each of the treatments are shown. (B) Quantitation of the ratio of nuclear GFP to cytoplasmic GFP by imaging analysis. *, $p < 0.01$.

a positive control (Schapira et al. 2003). The GR sequence anchors the protein in the cytoplasm in the absence of ligand for TR. Both a potent natural agonist, T3, and the antagonist, 1-850, stimulated nuclear translocation/stabilization of the GFP-GR-TRβ, as shown in Figure 2. We selected 285 compounds of the 699 candidate antagonists after initial filtering for cytotoxicity for testing in this assay. Selection was based on compound availability and potencies in the GH3-TRE-Antag assay while taking into account counter screening results for the GH3 cell viability assay, the cytotoxicity median AC_{50} value, and *in vitro* assay promiscuity rates in the nonviability Tox21 assays. Testing at a single, high concentration of 50 μM yielded 41 compounds that significantly increased nuclear translocation of the TR-GFP (Figure 3). These were retested in a concentration–response format, and results are shown in Figure 4 and supporting data in Excel Table S9. Most showed activity only at the highest concentrations and the results were considered equivocal. All compounds were also screened at the highest concentration in the parental MCF7 cell line without the GFP fusion protein in order to test for potential false-positive effects due to compound fluorescence. The 41 compounds considered active in the translocation assay did not show any evidence of assay interference (see Excel Table S9).

The summary of results for the potential TR antagonists is provided in Table 3. Examples of the different classes of response are shown in Figure 5 for a likely false positive due to cytotoxicity (Figure 5A), a probable false positive due to cytotoxicity but active in nuclear translocation (Figure 5B), and a likely true antagonist (Figure 5C). None of the compounds identified as candidate antagonists had been previously characterized as direct TR antagonists. The most active, carfilzomib, is an irreversible proteasome inhibitor approved for chemotherapy of multiple

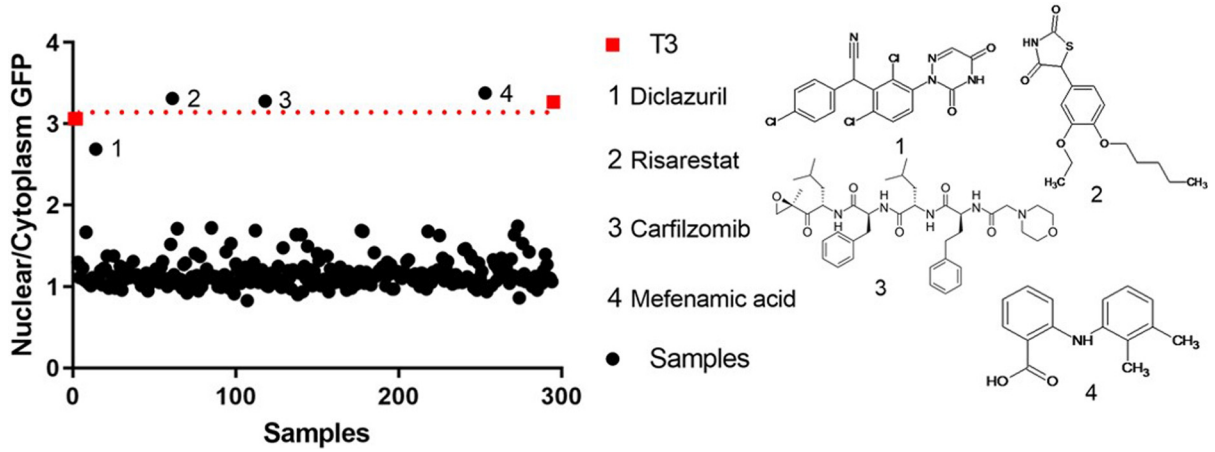

Figure 3. Nuclear translocation assay screening of 285 candidate thyroid hormone receptor (TR) antagonists. A total of 285 compounds were evaluated in the GFP-GR-TRβ translocation assay at a single concentration (100 μM). The positive control for translocation was triiodothyronine (T3) (100 nM) (red squares). An algorithm for cytoplasm and nuclear segmentation of the cells was used to determine the mean GFP-GR-TRβ intensity in both compartments, and translocation was quantified as a ratio of these intensities. Each value was normalized to the neutral control [dimethyl sulfoxide (DMSO)]. T3 produced a nuclear/cytoplasm ratio of 3, whereas inactive compounds were approximately 1. Several test compounds yielded similar results.

myeloma. It showed an AC_{50} of 25 nM for the GH3 assay for TR antagonist activity, similar to the active concentrations demonstrated for its proteasome inhibition and functional effects in myeloma cell lines (Kuhn et al. 2007). Carfilzomib is cytotoxic, active in 64% of Tox21 viability assays, and has a ratio of cell viability AC_{50} to GH3 transactivation AC_{50} of only 1.5. Thus, the effect in the translocation assay may be secondary to proteasome inhibition rather than direct TR activation. Another of the most active compounds in the nuclear translocation assay is mefenamic acid, a nonsteroidal inflammatory agent with very limited cytotoxicity in Tox21 assays (4.5% active rate). Mefenamic acid has been previously shown to displace T3 and T4 from plasma binding protein *in vivo* (Koizumi et al. 1984), from transthyretin isolated from human plasma *in vitro* (Munro et al. 1989), and from thyroid hormone binding proteins, such as transthyretin and albumin in placental cytosol (McKinnon et al. 2005). It can be inferred that the molecular shape of mefenamic acid may resemble that of T3 and T4 given that it can displace these ligands from their binding protein sites. Risarestat is a thiazolidinedione aldose reductase inhibitor and diclazuril is a broad-spectrum, anticoccidial drug with an undefined mechanism of action. Both lacked cytotoxicity in the GH3 viability assay and were modestly to moderately active in other Tox21 cytotoxicity assays, at 12% and 33% active, respectively. Both have an amine-dione structural feature that, in the case of risarestat, is part of the thiazolindinedione group, a known carboxylic acid–mimetic that participates in a critical hydrogen bond in peroxisome proliferator-activated receptor ligands (Falck et al. 2008). These structural features may be required to provide binding through a mechanism used by the carboxylic acid group of T3/T4 in hydrogen bonding to arginine residues, a key binding determinant for TR (Martínez et al. 2009). Indeed, Pfizer developed a 6-azauracil structural series highly related to diclazuril as TRβ subtype-selective thyromimetics (Dow et al. 2003). Daunorubicin was also very active in the nuclear translocation assay but was cytotoxic in two-thirds of the cytotoxicity/viability assays in which it was tested. Although positive in the translocation assay, daunorubicin significantly reduced average cell number per field in this high-content imaging assay, suggesting significant cytotoxicity. Thus, we considered it unlikely that the compound was acting directly on TR. Another active compound in the translocation assay that reduced average cell number per field was omacetaxine mepesuccinate, a natural product with chemotherapeutic properties via inhibition of protein translation (Gandhi et al. 2014). It also had a cytotoxicity AC_{50} twice as high as that of the TR reporter gene in GH3 cells, suggesting that direct action on TR is unlikely. Other compounds, less active in the translocation assay, were C.I. Basic Red 9 monohydrochloride, PP242, Methyl Red, lercanidipine hydrochloride, and ecopipam. These had cytotoxicity potencies less than 2-fold greater than the reporter gene, which may also indicate indirect effects on TR. Thus, only 3 of the top 12 candidate TR antagonists showing concentration-dependent effects on TR translocation appear to be potentially direct TR antagonists. Their structures and those of the other 9 are shown in Figure S3.

The three highest confidence antagonist compounds were evaluated for similarity to other compounds in the DSSTox list and the Tox21 library as was done with the agonists. There were 204 compounds identified in DSSTox (see Excel Table S10) but none were included in the Tox21 library. Of the 204 compounds, 34 were present on one or more lists included on the CompTox Chemicals Dashboard, and these may be of higher priority for follow-up work. It should be noted that the structure of each one of these three highest confidence antagonists had <0.8 Tanimoto similarity to the other two.

To put our screening results in context with other important endocrine nuclear receptors, we compared our TR screening results with those of estrogen and androgen receptors. A subset of the Tox21 library of approximately 1,600 chemicals were screened in numerous orthogonal assays for estrogen and androgen receptors, somewhat analogous to the follow-up assays performed for TR, and the data used in integrated pathway models predicting receptor activity. The active rate for these 1,600 chemicals for agonist and antagonist modes for estrogen receptor was 4.3% and 0.6%; for androgen receptor was 0.5% and 8.8%; and for TR 0.0% and 0.1%, respectively (see Excel Table S11) (Judson et al. 2015; Kleinstreuer et al. 2017).

Discussion

Understanding the interaction of xenobiotics with endocrine nuclear receptors is of critical importance for protecting the health of humans and wildlife. The U.S. EPA's EDSP was established to provide the means to test pesticides and other environmental chemicals for their potential to interact with the endocrine systems of human and wildlife. Much of this effort was initially

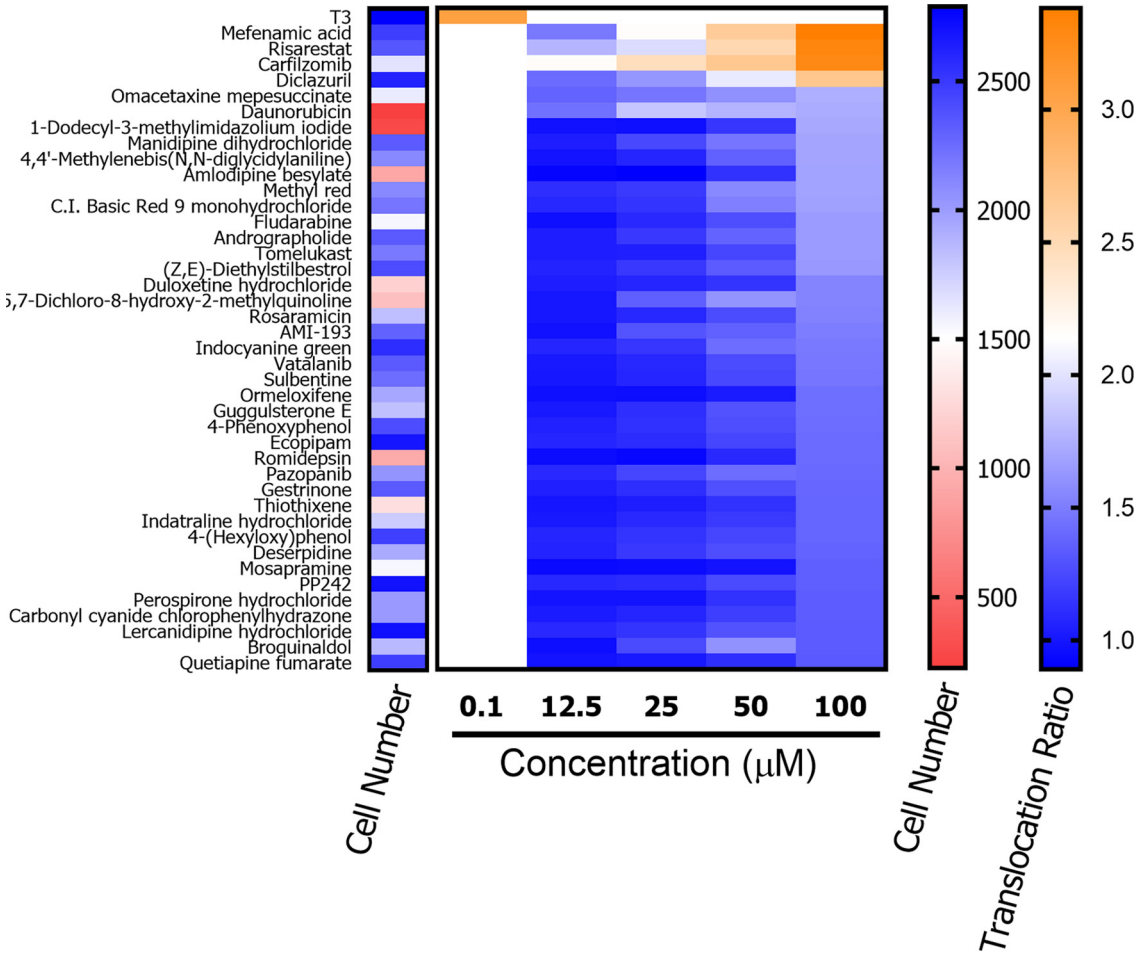

Figure 4. Multiple concentration testing of active compounds in the GFP-GR-TRβ translocation assay. The 41 compounds identified as active in the single concentration screen were retested at four concentrations in the GFP-GR-TRβ translocation assay. Data were analyzed and normalized as described in the text. Most compounds produced little or no concentration-dependent response. The ratio of the nuclear-to-cytoplasm fluorescence is presented in the heat map. The average cell number counted per well (10 images) is also indicated as a measure of potential cytotoxicity. Legends to each heat map are shown on the right. T3, triiodothyronine.

focused on screens and models for identifying xenobiotics targeting the estrogen and androgen receptors with numerous chemicals identified that bind and interfere with functions of these receptors (Browne et al. 2015; Kleinstreuer et al. 2017). The TR, no less a critical endocrine receptor, has been associated with only a small number of possible chemical modulator classes, such as the PBDEs and PCBs, suggesting a much more restrictive ligand-binding pocket (Meerts et al. 2001). We tested that hypothesis through a large-scale screen of an extensive collection of environmental chemicals for agonist or antagonist activity in a TR transactivation assay. Only 11 chemicals of 8,305 unique chemical structures were identified as direct TR ligands: 8 were agonists and 3 antagonists, supporting the conclusion that TR is a very restrictive receptor with limited ligand structural diversity.

Almost all the agonists, particularly the more efficacious agonists, are T3 analogs. Like T3, these compounds were both potent and displayed generally full activation of the receptor. Another class of active compounds was shown to agonize RXR through the TR:RXR heterodimer, resulting in partial agonist activity in the transactivation assays. This activity, called a permissive heterodimer effect, has been previously described (Castillo et al. 2004). As expected, these compounds were inactive in the coactivator recruitment assay given that no RXR was present. The physiological/toxicological relevance of this effect and whether xenobiotics could affect TR-regulated genes through RXR *in vivo* is unclear due to lack of published studies. Nevertheless, all the high confidence RXR agonists that modulated the TR reporter gene were pharmaceuticals (or naturally occurring substances used as pharmaceuticals). Thus, from the compounds tested, there were no environmental chemicals of potential concern with significant potency acting through this pharmacological mechanism although, as previously mentioned, hydroxylated polybrominated diphenyl ethers (PDBEs) were not part of the chemical library.

Identification of antagonists using the GH3-TRE-Luc reporter gene assay was much more challenging. Many compounds decreased cell viability at concentrations similar to their activity in the reporter gene assay, supporting the conclusion that the results are false positives with respect to TR antagonism. However, many other compounds, including examples of known cytotoxic and cytostatic ones, showed more potent effects against the reporter gene, which could be interpreted as true TR antagonism. It is important to consider, though, that the end points measured—a decrease in reporter gene activity versus a loss of a constitutive protease activity used for the cell viability measurement—are not necessarily coordinately linked either kinetically or quantitatively. Cytotoxicity mechanisms vary and can have very different kinetics; necrosis versus apoptosis as mechanisms of cell death serves as an example at a high level. Thus, chemicals could demonstrate different ratios of cytotoxic concentrations to the AC_{50} for inhibition of the reporter gene (Riss and Moravec 2004). An

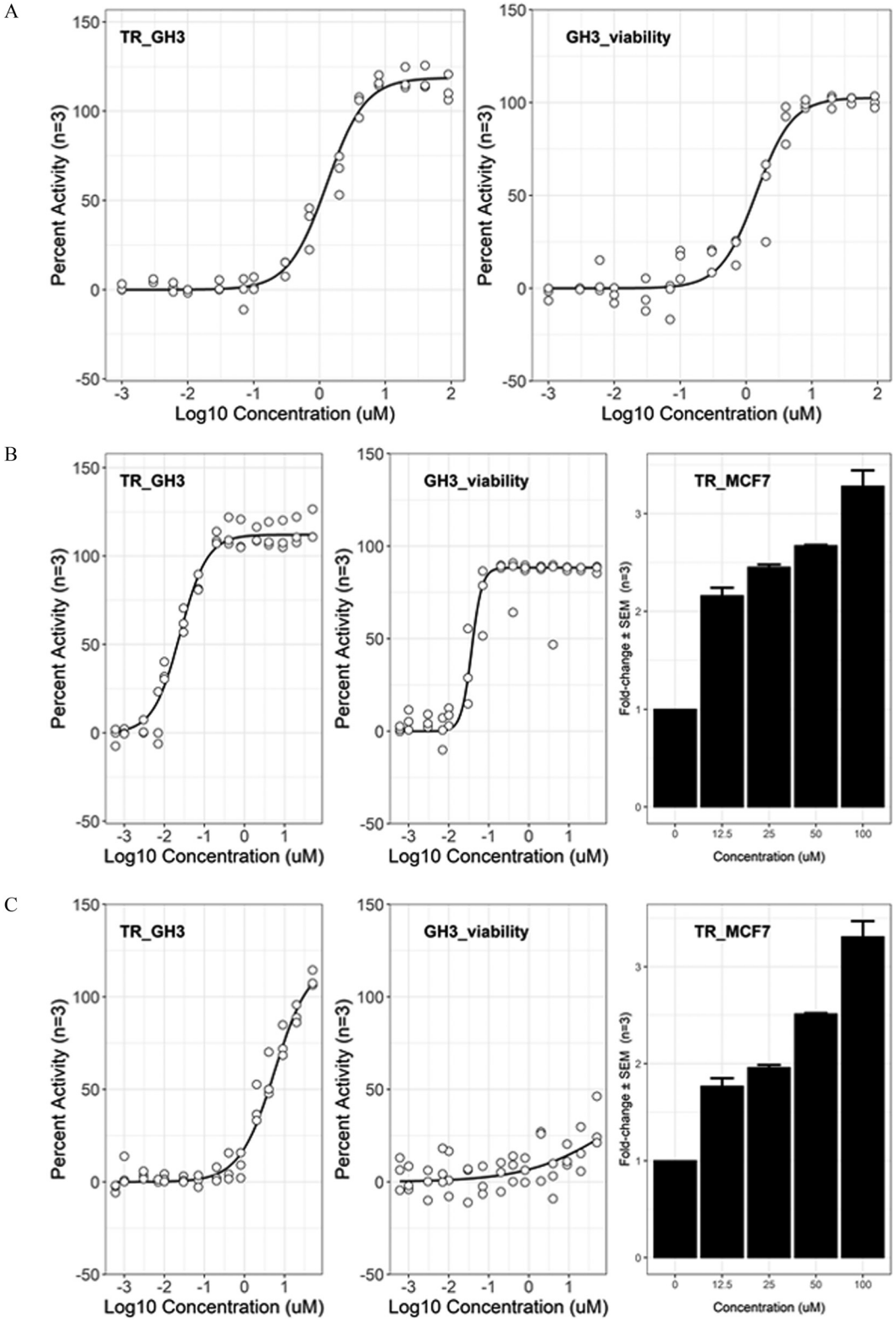

Figure 5. Example characterization of thyroid hormone receptor (TR) antagonists. Candidate TR antagonists were tested in concentration–response format in the TR_GH3 rat pituitary cell line used in the quantitative high-throughput screen (qHTS) concurrent with viability testing using the CellTiter-Glo reagent. (A) Dichlofluanid, an example of an active compound in the TR_Luc_GH3 antagonist mode assay; however, it showed loss of viability at virtually identical concentrations, suggesting the activity is likely due to cytotoxicity. (B) Carfilzomib, a selective proteasome inhibitor, was active in the TR_Luc_GH3 antagonist mode assay while causing loss of viability at slightly higher concentrations. It also showed concentration-dependent stimulation of GFP-GR-TRβ nuclear translocation and may be an indirect modulator of TR. (C) Risarestat, a thiazolidinedione aldose reductase inhibitor, was active in the TR_Luc_GH3 antagonist mode assay with little loss of viability and showed a concentration-dependent stimulation of GFP-GR-TRβ nuclear translocation and was classified as a likely direct TR antagonist.

orthogonal assay helped to confirm TR activity using a novel chimeric GR-TR nuclear receptor fused to a GFP that allows functional determination of ligand binding to the receptor through monitoring by automated fluorescent imaging of nuclear translocation/stabilization of the fusion protein. Most of the candidate antagonists were not active in the translocation assay, showing how confounding cytotoxicity or other interference mechanisms could be in reporter assay screens. There were 41 compounds that were scored as active at the highest concentration tested in the nuclear translocation assay and were negative in a background fluorescence counter screen. Of these, 16 produced concentration-dependent effects, increasing confidence in their activity as TR antagonists. The other 25 putative TR antagonists may be considered equivocal or only weakly active at high concentrations, with further testing required to confirm their activity.

The most active TR antagonists identified were all from the pharmaceutical use class of compounds. Mefenamic acid is an anthranilic acid and nonsteroidal anti-inflammatory drug (NSAID) (Paulus and Whitehouse 1973) with no reported previous characterization as a modulator of TR. However, it was previously shown to increase plasma T3 and T4 levels following an intravenous injection in rats, reportedly due to displacement of T3 and T4 from plasma binding protein, suggesting a similarity in molecular structure between mefenamic acid and T3 and T4 (Koizumi et al. 1984). Two drugs identified as TR antagonists in this work without previous evidence of TR activity are risarestat, an aldose reductase inhibitor developed as a treatment for hypoglycemia associated with diabetes, and diclazuril, an anticoccidial used in the poultry industry. The significance of these drugs having potential TR antagonist activity is unknown, but the potencies affecting TR activity are moderately high (ranging from 5–20 μM); as such, TR activity may occur at concentrations much higher than the systemic concentrations produced by therapeutic dosages. Nevertheless, this may warrant some investigation to understand whether such activities may be of pathophysiological relevance. Defining specific TR antagonists is challenging due to the potential for multiple mechanisms of TR modulation (i.e., indirect activity) and assay interference from multiple sources, including cytotoxicity. Omacetaxine mepesuccinate is a natural product oncolytic drug with a mechanism of action through inhibition of protein translation (Wetzler and Segal 2011) and is active nonspecifically in reporter gene assays. Activity of omacetaxine in the nuclear translocation assay could also be rationalized as an indirect effect, such as inhibition of the translation of chaperone proteins required to maintain the GFP-tagged human GR-TRβ hybrid receptor reporter protein in the cytoplasm. Thus, even with multiple characterization assays and manual curation, it is a challenge to identify a specific antagonist mechanism of action.

The overall conclusion of this large-scale screening of environmental and other chemicals for activity directly against the TR supports the hypothesis that TR is a very selective nuclear receptor. Others have described several notable classes of chemicals as TR modulators that were not reported here. Bisphenol A and brominated analogs have been characterized as TR antagonists (Kitamura et al. 2002; Moriyama et al. 2002). We found BPA (and BPA analogs) active in suppressing the TR transactivation assay; however, their activities were concurrent with cytotoxicity, suggesting that the observed effect on TR could be secondary to cytotoxicity. We have previously measured extensive BPA cytotoxicity and stress responses in the same concentration range and interpret these results as an indication of extensive cell stress, which can be manifested as promiscuous responses with *in vitro* assays (see Figure S4) (Judson et al. 2016). Likewise, the cardiac anti-arrhythmic amiodarone has been described as a TR antagonist but we, like others, found it cytotoxic at the concentrations at which it inhibits TR (Webb et al. 2002). Another class of compounds previously characterized as TR modulators are the PCBs and hydryoxylated PDBEs (Iwasaki et al. 2002; Kojima et al. 2009; Meerts et al. 2000; Zhou et al. 2002). None of the previously identified specific compounds were in the screening library, although four nonhydroxylated PCBs were included but were inactive against TR. In general, the physicochemical properties of PCBs are inconsistent with adequate aqueous solubility for *in vitro* testing, in particular for the nonhydroxylated compounds having high partition coefficient, whereas the hydroxylated analogs have limited commercial availability and high costs to procure.

Structural features of the TR suggest that the receptor may be restricted in the breadth of potential ligands recognized. Wagner et al. (1995) noted early on in studying the TR that the ligand–receptor interaction is very tight and that the ligand (i.e., T3) is completely buried in the ligand-binding pocket upon ligand-induced helix 12 conformational change. Subsequent attempts to develop TR agonists have relied upon a general design strategy based in solved crystal structures, yielding a pharmacophore model consisting of an acidic head group participating in a number of strong polar interactions with basic amino acid residues at one end of the binding pocket, a middle section characterized almost exclusively by hydrophobic interactions with the hydrophobic amino acids lining most of the pocket and including residues from helix 12, and a tail section with hydrogen bonding capability illustrated by the 4′ hydroxyl and 3′ steric iodine in the distal ring of T3 (Valadares et al. 2009). Selectivity between TRα and TRβ is difficult to achieve because there is only a single amino acid difference in the ligand-binding pocket (Huang et al. 2010). Specifically designed antagonists include protrusions from the mid-section that block helix 12 from obtaining a conformation that permits coactivator binding based on the extension hypothesis for nuclear receptor antagonists (Ribeiro et al. 1998; Webb et al. 2002). We note that our high confidence agonists and antagonists all meet most of these features. They contain either a carboxylic acid head group or a functionally similar structure capable of hydrogen bonding such as a thiazolidinedione or dioxytriazine. All have hydrophobic mid-sections. Note that although the endogenous ligands contain multiple iodines in this region involved in hydrophobic interactions and a halogen bond(s), their hydrophobicity can be replaced with methyl or dimethyl groups as in the potent agonist GC-1, which lacks iodine (Chiellini et al. 1998). It is interesting that in the major classes of nonpharmaceuticals previously reported as agonists or antagonists outside of this study (e.g., the PBDEs, PCBs, and bisphenols), the molecules lack an acidic head group. Given the promiscuity of at least some of these molecules, for example, tetrabromobisphenol A, it may suggest the carboxylate head group is critical for generating selectivity for TR. The novel candidate agonist betamipron contains a carboxylate head group but lacks an extensive hydrophobic middle, which likely contributes to its weak affinity. With respect to antagonists, the diclazuril structure contains a dioxytriazine head group likely participating in hydrogen bonding, a tri-halogenated biphenyl structure analogous to T3, and a nitrile group extension in the hydrophobic region. Although not necessarily considered a large protrusion as found in other nuclear receptor antagonists, it is a very polar group that possibly could destabilize hydrophobic helix 12 binding. We evaluated the structure using the similarity ensemble approach (SEA), which identifies putative small molecule targets through the structural similarity of a query structure to target ligands and found TRβ and TRα to be the top two matches with $p = 1.98 \times 10^{-20}$ and 7.1×10^{-10}, respectively (http://sea.bkslab.org/) (Keiser et al. 2007). Risarestat also mapped to TRα ($p = 9.99 \times 10^{-16}$) and TRβ

($p = 1.2 \times 10^{-12}$) using SEA. Most of the other candidate TR antagonists bear little resemblance to the T3 structure and are unlikely to directly bind to the LBD. They could, however, bind to the coactivator site to prevent agonist activity or work more indirectly, as previously mentioned, through mechanisms such as proteasome inhibition.

Although neurodevelopmental and other toxicities are of high concern for chemicals affecting the thyroid axis (Chen et al. 2016), it is likely that the molecular-initiating events that result in disruption of the thyroid axis occur through pathways beyond their direct effects on the TR. In contrast to the estrogen and androgen steroid hormone receptors, each affected by numerous environmental chemicals in the agonist or antagonist mode, respectively, TR behaves as a much more selective nuclear receptor for both agonist and antagonist modes of action. Indeed, we compared, for 1,600 chemicals, the chemical hit rate for estrogen and androgen receptors (0.5–8.8%, depending on receptor and mode) versus the more limited active rate for TR (0–0.1%, depending on mode) using the system of assays described herein; this suggested that even with the same chemical set, TR will be expected to be active at a lower rate than more permissive endocrine receptors. However, numerous mechanisms that alter levels of the endogenous hormones T3 and T4 may result in indirect effects, ultimately mediated through changed TR signaling levels and consequently pathological outcomes (Murk et al. 2013). Screening for disruptors of thyroid hormone function, regardless of mechanism, is critically important because maternal hypothyroxinemia correlates with irreversible neonatal cognitive and functional brain abnormalities (Barone et al. 2000; Berbel et al. 2009; Cuevas et al. 2005; Howdeshell 2002; Morreale de Escobar et al. 2000; Rice and Barone 2000; Zoeller and Crofton 2000), with decrements in intelligence quotient, cognition, socialization, and motor function following decreased maternal T4 (Haddow et al. 1999; Berbel et al. 2009; Kooistra et al. 2006; Li et al. 2010; Pop et al. 1999, 2003). Although transient disruption of thyroid hormone supply during neurodevelopment manifests in permanent alterations, the effects of thyroid hormone disruption in adults are generally reversible and treated pharmacologically (Biondi and Wartofsky 2014; Miller et al. 2009). It is well documented that T3 activates TRs to transcriptionally regulate gene expression required for myelination and neuronal and glial cell differentiation and migration in the developing brain during pregnancy and the early postnatal period (Bernal 2007; Clairman et al. 2015). Postnatally, neuronal cell migration in the hippocampus and cerebellum along with glial cell development and myelination also requires normal neonatal thyroid function (Porterfield and Hendrich 1993; Quignodon et al. 2004; Williams 2008). For example, experimentally induced hypo- and hyperthyroidism results in an altered external granular layer development in the rat cerebellar cortex at 10 d of age, probably through effects on the cell cycle (Lauder 1977) and, further, radiothyroidectomy of newborn rat pups resulted in failure of myelination of brain white matter (Rosman et al. 1972). More recent work in rats has demonstrated that hippocampal neurogenesis is reduced by developmental thyroid hormone disruption and that heterotopias are induced by chemically induced hypothyroidism (Gilbert et al. 2017; O'Shaughnessy et al. 2018). The clear connection between developmental thyroid hormone disruption and neurotoxicity underscores the need for a screening strategy to identify potential thyroid axis disrupting chemicals.

Because of its broad and critical importance, resources are being applied to better understand the major mechanisms and targets of environmental chemicals that may impact growth and development mediated by the thyroid axis beyond direct effects on the thyroid hormone receptors. Efforts in the U.S. EPA's ToxCast program have been initiated, including targeting and screening for inhibition of thyroperoxidase and thyroid uptake of iodide via the sodium-iodide symporter, both involved in thyroid hormone biosynthesis; inhibition of the MCT8 transmembrane transporter, which permits cellular uptake of thyroid hormones; and inhibition of human deiodinase type 1 (DIO1) and type 2 (DIO2) enzymes, which help maintain thyroid hormone homeostasis (Dong and Wade 2017; Hornung et al. 2018; Paul Friedman et al. 2016; Wang et al. 2018). Ongoing efforts in this area are needed to elucidate the molecular-initiating events of most relevance to thyroid hormone disruption by environmental exposures and, subsequently, to combine data from multiple molecular-initiating events for thyroid hormone disruption into outputs for screening-level chemical safety evaluation.

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References

Araki O, Ying H, Furuya F, Zhu X, Cheng SY. 2005. Thyroid hormone receptor β mutants: dominant negative regulators of peroxisome proliferator-activated receptor γ action. Proc Natl Acad Sci U S A 102(45):16251–16256, PMID: 16260719, https://doi.org/10.1073/pnas.0508556102.

Barone S Jr, Das KP, Lassiter TL, White LD. 2000. Vulnerable processes of nervous system development: a review of markers and methods. Neurotoxicology 21(1–2):15–36, PMID: 10794382.

Berbel P, Mestre JL, Santamaria A, Palazón I, Franco A, Graells M, et al. 2009. Delayed neurobehavioral development in children born to pregnant women with mild hypothyroxinemia during the first month of gestation: the importance of early iodine supplementation. Thyroid 19(5):511–519, PMID: 19348584, https://doi.org/10.1089/thy.2008.0341.

Berkenstam A, Kristensen J, Mellström K, Carlsson B, Malm J, Rehnmark S, et al. 2008. The thyroid hormone mimetic compound KB2115 lowers plasma LDL cholesterol and stimulates bile acid synthesis without cardiac effects in humans. Proc Natl Acad Sci U S A 105(2):663–667, PMID: 18160532, https://doi.org/10.1073/pnas.0705286104.

Bernal J. 2007. Thyroid hormone receptors in brain development and function. Nat Clin Pract Endocrinol Metab 3(3):249–259, PMID: 17315033, https://doi.org/10.1038/ncpendmet0424.

Biondi B, Wartofsky L. 2014. Treatment with thyroid hormone. Endocr Rev 35(3):433–512, PMID: 24433025, https://doi.org/10.1210/er.2013-1083.

Browne P, Judson RS, Casey WM, Kleinstreuer NC, Thomas RS. 2015. Screening chemicals for estrogen receptor bioactivity using a computational model. Environ Sci Technol 49(14):8804–8814, PMID: 26066997, https://doi.org/10.1021/acs.est.5b02641.

Burris TP, Solt LA, Wang Y, Crumbley C, Banerjee S, Griffett K, et al. 2013. Nuclear receptors and their selective pharmacologic modulators. Pharmacol Rev 65(2):710–778, PMID: 23457206, https://doi.org/10.1124/pr.112.006833.

Castillo AI, Sánchez-Martinez R, Moreno JL, Martínez-Iglesias OA, Palacios D, Aranda A. 2004. A permissive retinoid X receptor/thyroid hormone receptor heterodimer allows stimulation of prolactin gene transcription by thyroid hormone and 9-*cis*-retinoic acid. Mol Cell Biol 24(2):502–513, PMID: 14701725, https://doi.org/10.1128/MCB.24.2.502-513.2004.

Chen Q, Wang X, Shi W, Yu H, Zhang X, Giesy JP. 2016. Identification of thyroid hormone disruptors among Ho-PBDEs: *in vitro* investigations and coregulator involved simulations. Environ Sci Technol 50(22):12429–12438, PMID: 27737548, https://doi.org/10.1021/acs.est.6b02029.

Cheng SY, Leonard JL, Davis PJ. 2010. Molecular aspects of thyroid hormone actions. Endocr Rev 31(2):139–170, PMID: 20051527, https://doi.org/10.1210/er.2009-0007.

Chiellini G, Apriletti JW, Yoshihara HA, Baxter JD, Ribeiro RC, Scanlan TS. 1998. A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. Chem Biol 5(6):299–306, PMID: 9653548, https://doi.org/10.1016/S1074-5521(98)90168-5.

Chiellini G, Nguyen NH, Apriletti JW, Baxter JD, Scanlan TS. 2002. Synthesis and biological activity of novel thyroid hormone analogues: 5′-aryl substituted GC-1 derivatives. Bioorg Med Chem 10(2):333–346, PMID: 11741782, https://doi.org/10.1016/s0968-0896(01)00284-x.

Cioffi F, Gentile A, Silvestri E, Goglia F, Lombardi A. 2018. Effect of iodothyronines on thermogenesis: focus on brown adipose tissue. Front Endocrinol (Lausanne) 9:254, PMID: 29875734, https://doi.org/10.3389/fendo.2018.00254.

Clairman H, Skocic J, Lischinsky JE, Rovet J. 2015. Do children with congenital hypothyroidism exhibit abnormal cortical morphology? Pediatr Res 78(3):286–297, PMID: 25978801, https://doi.org/10.1038/pr.2015.93.

Crisman TJ, Parker CN, Jenkins JL, Scheiber J, Thoma M, Kang ZB, et al. 2007. Understanding false positives in reporter gene assays: in silico chemogenomics approaches to prioritize cell-based HTS data. J Chem Inf Model 47(4):1319–1327, PMID: 17608469, https://doi.org/10.1021/ci6005504.

Cuevas E, Ausó E, Telefont M, Morreale de Escobar G, Sotelo C, Berbel P. 2005. Transient maternal hypothyroxinemia at onset of corticogenesis alters tangential migration of medial ganglionic eminence-derived neurons. Eur J Neurosci 22(3):541–551, PMID: 16101736, https://doi.org/10.1111/j.1460-9568.2005.04243.x.

DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, et al. 1999. Screening methods for thyroid hormone disruptors. Environ Health Perspect 107(5):407–415, PMID: 10210697, https://doi.org/10.1289/ehp.99107407.

Dong H, Wade MG. 2017. Application of a nonradioactive assay for high throughput screening for inhibition of thyroid hormone uptake via the transmembrane transporter MCT8. Toxicol In Vitro 40:234–242, PMID: 28119167, https://doi.org/10.1016/j.tiv.2017.01.014.

Dow RL, Schneider SR, Paight ES, Hank RF, Chiang P, Cornelius P, et al. 2003. Discovery of a novel series of 6-azauracil-based thyroid hormone receptor ligands: potent, TRβ subtype-selective thyromimetics. Bioorg Med Chem Lett 13(3):379–382, PMID: 12565933, https://doi.org/10.1016/S0960-894X(02)00947-2.

Duncan Bassett JH, Williams GR. 2018. Analysis of physiological responses to thyroid hormones and their receptors in bone. Methods Mol Biol 1801:123–154, PMID: 29892822, https://doi.org/10.1007/978-1-4939-7902-8_12.

Falck JR, Gao S, Prasad RN, Koduru SR. 2008. Electrophilic α-thiocyanation of chiral and achiral *N*-acyl imides. A convenient route to 5-substituted and 5,5-disubstituted 2,4-thiazolidinediones. Bioorg Med Chem Lett 18(6):1768–1771, PMID: 18308568, https://doi.org/10.1016/j.bmcl.2008.02.034.

Filer DL, Kothiya P, Setzer RW, Judson RS, Martin MT. 2017. tcpl: the ToxCast pipeline for high-throughput screening data. Bioinformatics 33(4):618–620, PMID: 27797781, https://doi.org/10.1093/bioinformatics/btw680.

Freitas J, Cano P, Craig-Veit C, Goodson ML, Furlow JD, Murk AJ. 2011. Detection of thyroid hormone receptor disruptors by a novel stable *in vitro* reporter gene assay. Toxicol In Vitro 25(1):257–266, PMID: 20732405, https://doi.org/10.1016/j.tiv.2010.08.013.

Freitas J, Miller N, Mengeling BJ, Xia M, Huang R, Houck K, et al. 2014. Identification of thyroid hormone receptor active compounds using a quantitative high-throughput screening platform. Curr Chem Genom Transl Med 8:36–46, PMID: 24772387, https://doi.org/10.2174/2213988501408010036.

Gandhi V, Plunkett W, Cortes JE. 2014. Omacetaxine: a protein translation inhibitor for treatment of chronic myelogenous leukemia. Clin Cancer Res 20(7):1735–1740, PMID: 24501394, https://doi.org/10.1158/1078-0432.CCR-13-1283.

Gilbert ME, Goodman JH, Gomez J, Johnstone AFM, Ramos RL. 2017. Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption. Neurotoxicology 59:9–21, PMID: 28048979, https://doi.org/10.1016/j.neuro.2016.12.009.

Gilbert ME, Rovet J, Chen Z, Koibuchi N. 2012. Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences. Neurotoxicology 33(4):842–852, PMID: 22138353, https://doi.org/10.1016/j.neuro.2011.11.005.

Govindan M, Meng X, Denis CL, Webb P, Baxter JD, Walfish PG. 2009. Identification of CCR4 and other essential thyroid hormone receptor coactivators by modified yeast synthetic genetic array analysis. Proc Natl Acad Sci U S A 106(47):19854–19859, PMID: 19903885, https://doi.org/10.1073/pnas.0910134106.

Grijota-Martínez C, Samarut E, Scanlan TS, Morte B, Bernal J. 2011. *In vivo* activity of the thyroid hormone receptor β- and α-selective agonists GC-24 and CO23 on rat liver, heart, and brain. Endocrinology, PMID: 21239431, https://doi.org/10.1210/en.2010-0813.

Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, et al. 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. N Engl J Med 341(8):549–555, PMID: 10451459, https://doi.org/10.1056/NEJM199908193410801.

Hirouchi Y, Naganuma H, Kawahara Y, Okada R, Kamiya A, Inui K, et al. 1994. Preventive effect of betamipron on nephrotoxicity and uptake of carbapenems in rabbit renal cortex. Jpn J Pharmacol 66(1):1–6, PMID: 7861655, https://doi.org/10.1254/jjp.66.1.

Hofmann PJ, Schomburg L, Köhrle J. 2009. Interference of endocrine disrupters with thyroid hormone receptor-dependent transactivation. Toxicol Sci 110(1):125–137, PMID: 19403856, https://doi.org/10.1093/toxsci/kfp086.

Hornung MW, Korte JJ, Olker JH, Denny JS, Knutsen C, Hartig PC, et al. 2018. Screening the ToxCast phase 1 chemical library for inhibition of deiodinase type 1 activity. Toxicol Sci 162(2):570–581, PMID: 29228274, https://doi.org/10.1093/toxsci/kfx279.

Howdeshell KL. 2002. A model of the development of the brain as a construct of the thyroid system. Environ Health Perspect 110(Suppl 3):337–348, PMID: 12060827, https://doi.org/10.1289/ehp.02110s3337.

Htun H, Barsony J, Renyi I, Gould DL, Hager GL. 1996. Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. Proc Natl Acad Sci U S A 93(10):4845–4850, PMID: 8643491, https://doi.org/10.1073/pnas.93.10.4845.

Huang P, Chandra V, Rastinejad F. 2010. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol 72:247–272, PMID: 20148675, https://doi.org/10.1146/annurev-physiol-021909-135917.

Ibhazehiebo K, Iwasaki T, Okano-Uchida T, Shimokawa N, Ishizaki Y, Koibuchi N. 2011. Suppression of thyroid hormone receptor-mediated transcription and disruption of thyroid hormone-induced cerebellar morphogenesis by the polybrominated biphenyl mixture, BP-6. Neurotoxicology 32(4):400–409, PMID: 21396401, https://doi.org/10.1016/j.neuro.2011.02.008.

Iwasaki T, Miyazaki W, Takeshita A, Kuroda Y, Koibuchi N. 2002. Polychlorinated biphenyls suppress thyroid hormone-induced transactivation. Bioch Biophys Res Commun 299(3):384–388, PMID: 12445811, https://doi.org/10.1016/S0006-291X(02)02659-1.

Judson R, Houck K, Martin M, Richard AM, Knudsen TB, Shah I, et al. 2016. Analysis of the effects of cell stress and cytotoxicity on *in vitro* assay activity across a diverse chemical and assay space. Toxicol Sci 153(2):409, PMID: 27605417, https://doi.org/10.1093/toxsci/kfw148.

Judson RS, Magpantay FM, Chickarmane V, Haskell C, Tania N, Taylor J, et al. 2015. Integrated model of chemical perturbations of a biological pathway using 18 *in vitro* high-throughput screening assays for the estrogen receptor. Toxicol Sci 148(1):137–154, PMID: 26272952, https://doi.org/10.1093/toxsci/kfv168.

Kavlock RJ, Austin CP, Tice RR. 2009. Toxicity testing in the 21st century: implications for human health risk assessment. Risk Anal 29(4):485–487, PMID: 19076321, https://doi.org/10.1111/j.1539-6924.2008.01168.x.

Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK. 2007. Relating protein pharmacology by ligand chemistry. Nat Biotechnol 25(2):197–206, PMID: 17287757, https://doi.org/10.1038/nbt1284.

Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochem Biophys Res Commun 293(1):554–559, PMID: 12054637, https://doi.org/10.1016/S0006-291X(02)00262-0.

Kleinstreuer NC, Ceger P, Watt ED, Martin M, Houck K, Browne P, et al. 2017. Development and validation of a computational model for androgen receptor activity. Chem Res Toxicol, PMID: 27933809, https://doi.org/10.1021/acs.chemrestox.6b00347.

Koizumi Y, Sato A, Yamada T, Inada M. 1984. Effect of mefenamic acid on plasma protein-thyroid hormone interaction, monodeiodination of thyroxine, urinary excretion of tri-iodothyronine and thyrotropin regulation. Clin Exp Pharmacol Physiol 11(3):291–299, PMID: 6744687, https://doi.org/10.1111/j.1440-1681.1984.tb00267.x.

Kojima H, Takeuchi S, Uramaru N, Sugihara K, Yoshida T, Kitamura S. 2009. Nuclear hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in transactivation assays using Chinese hamster ovary cells. Environ Health Perspect 117(8):1210–1218, PMID: 19672399, https://doi.org/10.1289/ehp.0900753.

Kollitz EM, De Carbonnel L, Stapleton HM, Lee Ferguson P. 2018. The affinity of brominated phenolic compounds for human and zebrafish thyroid receptor β: influence of chemical structure. Toxicol Sci 163(1):226–239, PMID: 29409039, https://doi.org/10.1093/toxsci/kfy028.

Kooistra L, Crawford S, van Baar AL, Brouwers EP, Pop VJ. 2006. Neonatal effects of maternal hypothyroxinemia during early pregnancy. Pediatrics 117(1):161–167, PMID: 16396874, https://doi.org/10.1542/peds.2005-0227.

Kuhn DJ, Chen Q, Voorhees PM, Strader JS, Shenk KD, Sun CM, et al. 2007. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. Blood 110(9):3281–3290, PMID: 17591945, https://doi.org/10.1182/blood-2007-01-065888.

Lauder JM. 1977. The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. III. Kinetics of cell proliferation in the external granular layer. Brain Res 126(1):31–51, PMID: 856416, https://doi.org/10.1016/0006-8993(77)90213-X.

Lee H, Yen PM. 1999. Recent advances in understanding thyroid hormone receptor coregulators. J Biomed Sci 6(2):71–78, PMID: 10087437, https://doi.org/10.1007/BF02256437.

Li Y, Shan Z, Teng W, Yu X, Fan C, Teng X, et al. 2010. Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25–30 months. Clin Endocrinol (Oxf) 72(6):825–829, PMID: 19878506, https://doi.org/10.1111/j.1365-2265.2009.03743.x.

Lim W, Nguyen NH, Yang HY, Scanlan TS, Furlow JD. 2002. A thyroid hormone antagonist that inhibits thyroid hormone action *in vivo*. J Biol Chem 277(38):35664–35670, PMID: 12095994, https://doi.org/10.1074/jbc.M205608200.

Lonard DM, Lanz RB, O'Malley BW. 2007. Nuclear receptor coregulators and human disease. Endocr Rev 28(5):575–587, PMID: 17609497, https://doi.org/10.1210/er.2007-0012.

Martínez L, Nascimento AS, Nunes FM, Phillips K, Aparicio R, Dias SMG, et al. 2009. Gaining ligand selectivity in thyroid hormone receptors via entropy. Proc Natl Acad Sci U S A 106(49):20717–20722, PMID: 19926848, https://doi.org/10.1073/pnas.0911024106.

McKinnon B, Li H, Richard K, Mortimer R. 2005. Synthesis of thyroid hormone binding proteins transthyretin and albumin by human trophoblast. J Clin Endocrinol Metab 90(12):6714–6720, PMID: 16159939, https://doi.org/10.1210/jc.2005-0696.

Meerts IA, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, et al. 2001. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ Health Perspect 109(4):399–407, PMID: 11335189, https://doi.org/10.1289/ehp.01109399.

Meerts IA, van Zanden JJ, Luijks EAC, van Leeuwen-Bol I, Marsh G, Jakobsson E, et al. 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. Toxicol Sci 56(1):95–104, PMID: 10869457, https://doi.org/10.1093/toxsci/56.1.95.

Miller MD, Crofton KM, Rice DC, Zoeller RT. 2009. Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. Environ Health Perspect 117(7):1033–1041, PMID: 19654909, https://doi.org/10.1289/ehp.0800247.

Miyazaki W, Iwasaki T, Takeshita A, Tohyama C, Koibuchi N. 2008. Identification of the functional domain of thyroid hormone receptor responsible for polychlorinated biphenyl-mediated suppression of its action *in vitro*. Environ Health Perspect 116(9):1231–1236, PMID: 18795168, https://doi.org/10.1289/ehp.11176.

Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, et al. 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 87(11):5185–5190, PMID: 12414890, https://doi.org/10.1210/jc.2002-020209.

Morreale de Escobar G, Obregón MJ, Escobar del Rey F. 2000. Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia? J Clin Endocrinol Metab 85(11):3975–3987, PMID: 11095417, https://doi.org/10.1210/jcem.85.11.6961.

Munro SL, Lim CF, Hall JG, Barlow JW, Craik DJ, Topliss DJ, et al. 1989. Drug competition for thyroxine binding to transthyretin (prealbumin): comparison with effects on thyroxine-binding globulin. J Clin Endocrinol Metab 68(6):1141–1147, PMID: 2498384, https://doi.org/10.1210/jcem-68-6-1141.

Murk AJ, Rijntjes E, Blaauboer BJ, Clewell R, Crofton KM, Dingemans MM, et al. 2013. Mechanism-based testing strategy using *in vitro* approaches for identification of thyroid hormone disrupting chemicals. Toxicol In Vitro 27(4):1320–1346, PMID: 23453986, https://doi.org/10.1016/j.tiv.2013.02.012.

Norman MF, Lavin TN. 1989. Antagonism of thyroid hormone action by amiodarone in rat pituitary tumor cells. J Clin Invest 83(1):306–313, PMID: 2910914, https://doi.org/10.1172/JCI113874.

O'Shaughnessy KL, Kosian PA, Ford JL, Oshiro WM, Degitz SJ, Gilbert ME. 2018. Developmental thyroid hormone insufficiency induces a cortical brain malformation and learning impairments: a cross-fostering study. Toxicol Sci 163(1):101–115, PMID: 29385626, https://doi.org/10.1093/toxsci/kfy016.

Oetting A, Yen PM. 2007. New insights into thyroid hormone action. Best Pract Res Clin Endocrinol Metab 21(2):193–208, PMID: 17574003, https://doi.org/10.1016/j.beem.2007.04.004.

Paul Friedman K, Watt ED, Hornung MW, Hedge JM, Judson RS, Crofton KM, et al. 2016. Tiered high-throughput screening approach to identify thyroperoxidase inhibitors within the ToxCast phase I and II chemical libraries. Toxicol Sci 151(1):160–180, PMID: 26884060, https://doi.org/10.1093/toxsci/kfw034.

Paulus HE, Whitehouse MW. 1973. Nonsteroid anti-inflammatory agents. Annu Rev Pharmacol 13:107–125, PMID: 4576885, https://doi.org/10.1146/annurev.pa.13.040173.000543.

Pop VJ, Brouwers EP, Vader HL, Vulsma T, van Baar AL, de Vijlder JJ. 2003. Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. Clin Endocrinol (Oxf) 59(3):282–288, PMID: 12919150, https://doi.org/10.1046/j.1365-2265.2003.01822.x.

Pop VJ, Kuijpens JL, van Baar AL, Verkerk G, van Son MM, de Vijlder JJ, et al. 1999. Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. Clin Endocrinol (Oxf) 50(2):149–155, PMID: 10396355, https://doi.org/10.1046/j.1365-2265.1999.00639.x.

Porterfield SP, Hendrich CE. 1993. The role of thyroid hormones in prenatal and neonatal neurological development—current perspectives. Endocr Rev 14:94–106, PMID: 8491157, https://doi.org/10.1210/edrv-14-1-94.

Quignodon L, Legrand C, Allioli N, Guadaño-Ferraz A, Bernal J, Samarut J, et al. 2004. Thyroid hormone signaling is highly heterogeneous during pre- and postnatal brain development. J Mol Endocrinol 33(2):467–476, PMID: 15525602, https://doi.org/10.1677/jme.1.01570.

Ribeiro RC, Apriletti JW, Wagner RL, West BL, Feng W, Huber R, et al. 1998. Mechanisms of thyroid hormone action: insights from X-ray crystallographic and functional studies. Recent Prog Horm Res 53:351–392, PMID: 9769715.

Rice D, Barone S Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(Suppl 3):511–533, PMID: 10852851, https://doi.org/10.1289/ehp.00108s3511.

Riss TL, Moravec RA. 2004. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay Drug Dev Technol 2(1):51–62, PMID: 15090210, https://doi.org/10.1089/154065804322966315.

Rosman NP, Malone MJ, Helfenstein M, Kraft E. 1972. The effect of thyroid deficiency on myelination of brain. A morphological and biochemical study. Neurology 22(1):99–106, PMID: 5061842, https://doi.org/10.1212/wnl.22.1.99.

Schapira M, Raaka BM, Das S, Fan L, Totrov M, Zhou Z, et al. 2003. Discovery of diverse thyroid hormone receptor antagonists by high-throughput docking. Proc Natl Acad Sci U S A 100(12):7354–7359, PMID: 12777627, https://doi.org/10.1073/pnas.1131854100.

Schriks M, Vrabie CM, Gutleb AC, Faassen EJ, Rietjens IM, Murk AJ. 2006. T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs). Toxicol In Vitro 20(4):490–498, PMID: 16219445, https://doi.org/10.1016/j.tiv.2005.09.001.

Stanton JL, Cahill E, Dotson R, Tan J, Tomaselli HC, Wasvary JM, et al. 2000. Synthesis and biological activity of phenoxyphenyl oxamic acid derivatives related to L-thyronine. Bioorg Med Chem Lett 10(15):1661–1663, PMID: 10937719, https://doi.org/10.1016/S0960-894X(00)00309-7.

Stavreva DA, Varticovski L, Levkova L, George AA, Davis L, Pegoraro G, et al. 2016. Novel cell-based assay for detection of thyroid receptor beta-interacting environmental contaminants. Toxicology 368–369:69–79, PMID: 27528272, https://doi.org/10.1016/j.tox.2016.08.012.

Tancevski I, Rudling M, Eller P. 2011. Thyromimetics: a journey from bench to bedside. Pharmacol Ther 131(1):33–39, PMID: 21504761, https://doi.org/10.1016/j.pharmthera.2011.04.003.

Thorne N, Auld DS, Inglese J. 2010. Apparent activity in high-throughput screening: origins of compound-dependent assay interference. Curr Opin Chem Biol 14(3):315–324, PMID: 20417149, https://doi.org/10.1016/j.cbpa.2010.03.020.

Valadares NF, Salum LB, Polikarpov I, Andricopulo AD, Garratt RC. 2009. Role of halogen bonds in thyroid hormone receptor selectivity: pharmacophore-based 3D-QSSR studies. J Chem Inf Model 49(11):2606–2616, PMID: 19863110, https://doi.org/10.1021/ci900316e.

Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ. 1995. A structural role for hormone in the thyroid hormone receptor. Nature 378(6558):690–697, PMID: 7501015, https://doi.org/10.1038/378690a0.

Wallis K, Dudazy S, van Hogerlinden M, Nordström K, Mittag J, Vennström B. 2010. The thyroid hormone receptor α1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. Mol Endocrinol 24(10):1904–1916, PMID: 20739404, https://doi.org/10.1210/me.2010-0175.

Wang J, Hallinger DR, Murr AS, Buckalew AR, Simmons SO, Laws SC, et al. 2018. High-throughput screening and quantitative chemical ranking for sodium-iodide symporter inhibitors in ToxCast phase I chemical library. Environ Sci Technol 52(9):5417–5426, PMID: 29611697, https://doi.org/10.1021/acs.est.7b06145.

Wang M, Shim JS, Li RJ, Dang Y, He Q, Das M, et al. 2014. Identification of an old antibiotic clofoctol as a novel activator of unfolded protein response pathways and an inhibitor of prostate cancer. Br J Pharmacol 171(19):4478–4489, PMID: 24903412, https://doi.org/10.1111/bph.12800.

Wang Y, Huang R. 2016. Correction of microplate data from high-throughput screening. Methods Mol Biol 1473:123–134, PMID: 27518630, https://doi.org/10.1007/978-1-4939-6346-1_13.

Webb P, Nguyen N-H, Chiellini G, Yoshihara HAI, Cunha Lima ST, Apriletti JW, et al. 2002. Design of thyroid hormone receptor antagonists from first principles. J Steroid Biochem Mol Biol 83(1–5):59–73, PMID: 12650702, https://doi.org/10.1016/S0960-0760(02)00270-4.

Wetzler M, Segal D. 2011. Omacetaxine as an anticancer therapeutic: what is old is new again. Curr Pharm Des 17(1):59–64, PMID: 21294709, https://doi.org/10.2174/138161211795049778.

Williams GR. 2008. Neurodevelopmental and neurophysiological actions of thyroid hormone. J Neuroendocrinol 20(6):784–794, PMID: 18601701, https://doi.org/10.1111/j.1365-2826.2008.01733.x.

Yen PM. 2001. Physiological and molecular basis of thyroid hormone action. Physiol Rev 81(3):1097–1142, PMID: 11427693, https://doi.org/10.1152/physrev.2001.81.3.1097.

Zhou T, Taylor MM, DeVito MJ, Crofton KM. 2002. Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. Toxicol Sci 66(1):105–116, PMID: 11861977, https://doi.org/10.1093/toxsci/66.1.105.

Zoeller RT. 2005. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? Mol Cell Endocrinol 242(1–2):10–15, PMID: 16150534, https://doi.org/10.1016/j.mce.2005.07.006.

Zoeller RT, Crofton KM. 2000. Thyroid hormone action in fetal brain development and potential for disruption by environmental chemicals. Neurotoxicology 21(6):935–945, PMID: 11233763.

Zoeller RT, Tan SW, Tyl RW. 2007. General background on the hypothalamic-pituitary-thyroid (HPT) axis. Crit Rev Toxicol 37(1–2):11–53, PMID: 17364704, https://doi.org/10.1080/10408440601123446.